VERIFICATION OF TRANSLATION

I, Akiko Maruyama

of c/o KYOWA HAKKO KOGYO CO., LTD., 6-1, Ohtemachi 1-chome, Chiyoda-ku, Tokyo 100-8185 Japan

declare as follows:

1. That I am well acquainted with both the English and Japanese languages, and

2. That the attached document is a true and correct translation made by me to the best of my knowledge and belief of:

the specification accompanying the Application No. 2001-400677 for an application filed in Japan on December 28, 2001

January 30, 2008
(Date)

Akiko Maruyama
(Signature of Translator)

PATENT OFFICE
JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this Office

DATE OF APPLICATION: December 28, 2001

APPLICATION NUMBER: 2001-400677
[ST.10/C]: [JP2001-4006777]

APPLICANT(S) KYOWA HAKKO KOGYO CO., LTD.

Dated this 12th day of February, 2003

Shinichiro Ota
Commissioner,
Japan Patent Office

Certified No.:2003-3006018

[Name of Document]

Application for Patent

[Reference Number]

H13-2344S3

[Filing Date]

December 28, 2001

[Addressee]

Commissioner, Japan Patent Office

[International Patent Classification]

A61K 39/00 ABG

A61P 19/02

C12P 21/08

[Inventor]

[Address] c/o Pharmaceutical Research Institute, KYOWA HAKKO KOGYO CO., LTD., 1188, Shimotogari, Nagaizumi-cho, Sunto-gun Shizuoka

[Name] Tadafumi Tamura

[Inventor]

[Address] c/o Pharmaceutical Research Institute, KYOWA HAKKO KOGYO CO., LTD., 1188, Shimotogari, Nagaizumi-cho, Sunto-gun Shizuoka

[Name] Masako Uchii

[Inventor]

[Address] c/o Pharmaceutical Research Institute, KYOWA HAKKO KOGYO CO., LTD., 1188, Shimotogari, Nagaizumi-cho, Sunto-gun Shizuoka

[Name] Ichiro Miki

[Inventor]

[Address] 3-1-3-D-303, Gion, Minamikawachi-machi, Kawachi-gun, Tochigi

[Name] Akira Tanaka

[Applicant]

[Identification Number] 000001029

[Name] Kyowa Hakko Kogyo Co., Ltd.

[Representative] Tadashi Hirata

[Identification of Fees]

[Prepayment Book Number] 008187

[Amount of Payment] ¥21,000

[List of Submitted Articles]

[Name of Article] Specification 1 copy

[Name of Article] Drawing 1 copy

[Name of Article] Abstract 1 copy

[Necessity of Proofing] Yes

[Name of Document] Specification

[Title of the invention] AGENT FOR TREATING ARTHRITIS

[Scope of Claim for Patent]

[Claim 1] An agent for preventing or treating arthritis, comprising, as an active ingredient, an antibody which specifically binds to FGF-8 to inhibit activity of FGF-8.

[Claim 2] The agent according to claim 1, wherein the antibody which specifically binds to FGF-8 to inhibit activity of FGF-8 is a monoclonal antibody.

[Claim 3] The agent according to claim 2, wherein the monoclonal antibody is an antibody selected from an antibody produced by a hybridoma, a humanized antibody and an antibody fragment thereof.

[Claim 4] The agent according to claim 3, wherein the hybridoma is hybridoma KM1334 (FERM BP-5451).

[Claim 5] The agent according to claim 3, wherein the humanized antibody is an antibody selected from a human chimeric antibody and a human complementarity determining region (CDR)-grafted antibody.

[Claim 6] The agent according to claim 5, wherein the human chimeric antibody is an antibody in which an antibody heavy chain variable region (VH) comprises an amino acid sequence represented by SEQ ID NO. 2, and/or an antibody light chain variable region (VL) comprises an amino acid sequence represented by SEQ ID NO. 4.

[Claim 7] The agent according to claim 6, wherein the human chimeric antibody is a human chimeric antibody produced by transformant KM3034 (FERM BP-7836).

[Claim 8] The agent according to claim 5, wherein the human CDR-grafted antibody is an antibody in which CDR1, CDR2 and CDR3 of VH comprise amino acid sequences represented by SEQ ID NOS. 5, 6 and 7 respectively, and/or CDR1, CDR2 and CDR3 of VL comprise amino acid sequences represented by SEQ ID NOS. 8, 9 and 10 respectively.

[Claim 9] The agent according to claim 8, wherein the human CDR-grafted antibody is an antibody in which VH comprises amino

acid sequence represented by SEQ ID NO. 15 and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16.

[Claim 10] The agent according to claim 8, wherein the human CDR-grafted antibody is an antibody in which VH comprises an amino acid sequence represented by SEQ ID NO. 15 in which at least one or more amino acid residue selected from Lys at position 12, Lys at position 13, Ala at position 40, Pro at position 41, Met at position 48, Val at position 68, Ile at position 70, Thr at position 74, Thr at position 76, Glu at position 82, Ser at position 84, Arg at position 87 and Tyr at position 95 is replaced with another amino acid residue, and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16 in which at least one or more amino acid residue selected from Ile at position 2, Val at position 3, Thr at position 14, Pro at position 15, Gln at position 50, Leu at position 51 and Tyr at position 92 is replaced with another amino acid residue.

[Claim 11] The agent according to claim 3, wherein the antibody fragment is an antibody fragment selected from Fab, Fab', $F(ab')_2$, a single chain antibody (scFv), a dimerized variable region (V region) fragment (diabody), a disulfide-stabilized V region fragment (dsFv) and a CDR-containing peptide.

[Claim 12] A diagnostic agent of arthritis comprising an antibody which specifically binds to FGF-8 as an active ingredient.

[Claim 13] The diagnostic agent according to claim 12, wherein the antibody which specifically binds to FGF-8 is a polyclonal antibody or a monoclonal antibody.

[Claim 14] The diagnostic agent according to claim 13, wherein the monoclonal antibody is an antibody selected from an antibody produced by a hybridoma, a humanized antibody and an antibody fragment thereof.

[Claim 15] The diagnostic agent according to claim 14, wherein the hybridoma is hybridoma KM1334 (FERM BP-5451).

[Claim 16] The diagnostic agent according to claim 14, wherein the humanized antibody is an antibody selected from

a human chimeric antibody and a human CDR-grafted antibody.

[Claim 17] The diagnostic agent according to claim 16, wherein the human chimeric antibody is an antibody in which VH comprises amino acid sequence represented by SEQ ID NO. 2 and/or VL comprises an amino acid sequence represented by SEQ ID NO. 4.

[Claim 18] The diagnostic agent according to claim 17, wherein the human chimeric antibody is a human chimeric antibody produced by transformant KM3034 (FERM BP-7836).

[Claim 19] The diagnostic agent according to claim 16, wherein the human CDR-grafted antibody is an antibody in which CDR1, CDR2 and CDR3 of VH comprise amino acid sequences represented by SEQ ID NOS. 5, 6 and 7 respectively, and/or CDR1, CDR2 and CDR3 of VL comprise amino acid sequences represented by SEQ ID NOS. 8, 9 and 10 respectively.

[Claim 20] The diagnostic agent according to claim 19, wherein the human CDR-grafted antibody is an antibody in which VH comprises amino acid sequence represented by SEQ ID NO. 15 and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16.

[Claim 21] The diagnostic agent according to claim 19, wherein the human CDR-grafted antibody is an antibody in which VH comprises an amino acid sequence represented by SEQ ID NO. 15 in which at least one or more amino acid residue selected from Lys at position 12, Lys at position 13, Ala at position 40, Pro at position 41, Met at position 48, Val at position 68, Ile at position 70, Thr at position 74, Thr at position 76, Glu at position 82, Ser at position 84, Arg at position 87 and Tyr at position 95 is replaced with another amino acid residue, and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16 in which at least one or more amino acid residue selected from Ile at position 2, Val at position 3, Thr at position 14, Pro at position 15, Gln at position 50, Leu at position 51 and Tyr at position 92 is replaced with another amino acid residue.

[Claim 22] The diagnostic agent according to claim 14,

wherein the antibody fragment is an antibody fragment selected from Fab, Fab', $F(ab')_2$, a single chain antibody (scFv), a dimerized V region fragment (diabody), a disulfide-stabilized V region fragment (dsFv) and a CDR-containing peptide.

[Claim 23] A diagnostic method for judging arthritis, which comprises detecting and/or determining FGF-8 in a sample using an antibody which specifically binds to FGF-8.

[Claim 24] The diagnostic method according to claim 23, wherein the antibody which specifically binds to FGF-8 is a polyclonal antibody or a monoclonal antibody.

[Claim 25] The diagnostic method according to claim 24, wherein the monoclonal antibody is an antibody selected from an antibody produced by a hybridoma, a humanized antibody and an antibody fragment thereof.

[Claim 26] The diagnostic method according to claim 25, wherein the hybridoma is hybridoma KM1334 (FERM BP-5451).

[Claim 27] The diagnostic method according to claim 25, wherein the humanized antibody is an antibody selected from a human chimeric antibody and a human CDR-grafted antibody.

[Claim 28] The diagnostic method according to claim 27, wherein the human chimeric antibody is an antibody in which VH comprises amino acid sequence represented by SEQ ID NO. 2 and/or VL comprises an amino acid sequence represented by SEQ ID NO. 4.

[Claim 29] The diagnostic method according to claim 28, wherein the human chimeric antibody is a human chimeric antibody produced by transformant KM3034 (FERM BP-7836).

[Claim 30] The diagnostic method according to claim 27, wherein the human CDR-grafted antibody is an antibody in which CDR1, CDR2 and CDR3 of VH comprise amino acid sequences represented by SEQ ID NOS. 5, 6 and 7 respectively, and/or CDR1, CDR2 and CDR3 of VL comprise amino acid sequences represented by SEQ ID NOS. 8, 9 and 10 respectively.

[Claim 31] The diagnostic method according to claim 30, wherein the human CDR-grafted antibody is an antibody in which VH comprises amino acid sequence represented by SEQ ID NO. 15

and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16.

[Claim 32] The diagnostic method according to claim 30, wherein the human CDR-grafted antibody is an antibody in which VH comprises an amino acid sequence represented by SEQ ID NO. 15 in which at least one or more amino acid residue selected from Lys at position 12, Lys at position 13, Ala at position 40, Pro at position 41, Met at position 48, Val at position 68, Ile at position 70, Thr at position 74, Thr at position 76, Glu at position 82, Ser at position 84, Arg at position 87 and Tyr at position 95 is replaced with another amino acid residue, and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16 in which at least one or more amino acid residue selected from Ile at position 2, Val at position 3, Thr at position 14, Pro at position 15, Gln at position 50, Leu at position 51 and Tyr at position 92 is replaced with another amino acid residue.

[Claim 33] The diagnostic method according to claim 25, wherein the antibody fragment is an antibody fragment selected from Fab, Fab', $F(ab')_2$, a single chain antibody (scFv), a dimerized V region fragment (diabody), a disulfide-stabilized V region fragment (dsFv) and a CDR-containing peptide.

[Claim 34] An agent for inhibiting joint destruction comprising, as an active ingredient, an antibody which specifically binds to FGF-8 to inhibit activity of FGF-8.

[Claim 35] An agent for protecting cartilage comprising, as an active ingredient, an antibody which specifically binds to FGF-8 to inhibit activity of FGF-8.

[Claim 36] An agent for inhibiting growth of synovial membrane comprising, as an active ingredient, an antibody which specifically binds to FGF-8 to inhibit activity of FGF-8.

[Detailed Description of the Invention]

[Technical Field of the Invention]

The present invention relates to an agent for preventing or treating arthritis, an agent for inhibiting joint destruction,

an agent for protecting cartilage, an agent for inhibiting growth of synovial membrane and a diagnostic agent for arthritis comprising an anti-FGF-8 antibody as an active ingredient, as well as a diagnostic method for judging arthritis using the antibody.

[Prior Art]

The number of persons complaining of arthropathy has been surely increased in the aging society. It is very important to perform early diagnosis or screening of diseases such as osteoarthritis and rheumatoid arthritis as typical articular diseases or exact prognostic analysis of patients, and the treatment thereof leads to the improve in quality of life of many aged persons. However, satisfactory diagnostic and therapeutic methods have not yet been established.

The articular cartilage is a tissue that comprises a small number of chondrocytes covering the movable surface of the joint and a large number of extracellular matrix. Blood vessels or nerves are not distributed therein, and nutrients are supplied mainly from a synovial fluid produced from the synovial membrane covering the inner surface of the joint. Further, it is not only avascular but also exhibits strong resistance to invasion of blood vessels from the peripheral tissues rich in vasoganglion. Chondrocytes intricately control both of synthesis and degradation of extracellular matrix to play a major role in maintaining homeostasis of extracellular matrix. Chemical factors such as cytokines and growth factors and dynamic factors such as weight loading act on chondrocytes and change the balance of both the synthesis and the degradation of extracellular matrix to influence metabolism of extracellular matrix.

Osteoarthritis is caused by the aging or mechanical stresses to thereby induce disruption of the articular cartilage surface accompanied by growth of new cartilages around joints, deformation of joints and failure of adaptability and to lead to inflammation of synovial membranes of joints. The osteoarthritis is a monoarthritis disease with delayed

denaturation of the articular cartilage, and characteristics thereof are often pains and functional loss (Manek M. J. and Lane N. E., Am. Fam. Physician, 61, 1795-1804, 2000).

In rheumatoid arthritis, inflammatory cells invade synovial membranes because of immunological abnormality or infectious diseases, and the growth of synovial fibroblasts progresses according to angiogenesis to form an inflammatory synovial granulation tissue called pannus. When the pannus is formed, destruction of bones or cartilages proceeds to cause irreversible disorder in joints. During the destruction of bones or cartilages, various extracellular matrix present in large quantities, such as collagen and proteoglycan are degraded.

In articular diseases such as osteoarthritis and rheumatoid arthritis, the synovitis and the destruction of extracellular matrix lead to the functional loss of articular cartilages.

Osteoarthritis and rheumatoid arthritis are quite different diseases, but have many common points in the articular cartilage destruction mechanism. Many types of matrix metalloproteases are produced and secreted in the articular synovial fluid and articular portions such as a synovial membrane and a cartilage, and matrix metalloproteases are excessively detected in the articular portions. Matrix metalloproteases degrade many types of extracellular matrix, which is one cause of articular destruction. They are produced not only from inflamed synovial membranes, macrophages and neutrophils but also from chondrocytes. This production is controlled by various cytokines produced or secreted in the same articular portions, superoxide anion, nitric oxide, prostaglandins, growth factors and the like. It has been reported that these induce the production of matrix metalloproteases from synovial cells and chondrocytes to promote degradation of extracellular matrix.

From these reports, it is considered that osteoarthritis and rheumatoid arthritis as well as arthritic diseases such

as systemic lupus erythematosus which is an cryptogenic disease with an inflammatory tissue disorder caused by appearance of autoantibody and tissue deposition of an antigen-antibody complex and in which arthropathy occurs at a high rate, arthropathy, psoriatic arthritis leading to bone destruction with synovial membrane growth complicated in psoriatic patients, discopathy in which destruction of extracellular matrix of the intervertebral disc disease is observed and acute crystalline synovitis (gout, pseudogout) (Ryumachi Gaku, compiled by Hirohata Kazushi et al., Dobun Shoin, 1989) can be treated by inhibiting growth of the synovial membrane or destruction of cartilages.

In the pharmacotherapy of rheumatoid arthritis, various non-steroidal antiinflammatory agents, steroidal agents such as prednisolone and antirheumatic agents such as methotrexate have been so far used mainly to reduce pains and inflammation of joints (Chiryo, 78, 3553-3558, Nanzando, 1996). In osteoarthritis, various non-steroidal antiinflammatory agents, analgesic agents, hyaluronic acid pharmaceutical preparations as an intraarticular injection and the like have been administered to remove pains and inflammation. Hyaluronic acid which inhibits destruction of cartilages has been used as an agent for protecting cartilage (Creamer P., J. Rheum., 20, 1461-1464, 1993, Arthritis Rheum., 43, 1905-1915, 2000). Further, physical therapy and operative treatments such as osteotomy and artificial joint replacement have been carried out. Non-steroidal antiinflammatory agents and steroidal agents such as prednisolone are used in systemic lupus erythematosus, non-steroidal antiinflammatory agents and sulfasalazine as an antirheumatic drug in ankylotic arthropathy, non-steroidal antiinflammatory agents, antirheumatic drugs and steroidal intraarticular injections in psoriatic arthritis which involves synovial membrane growth complicated in psoriatic patients and leads to bone destruction, non-steroidal antiinflammatory agents and analgesic agents in intervertebral disc disease in which destruction of extracellular matrix of

the intervertebral disk is observed, and non-steroidal antiinflammatory agents, colchicine and the like in acute crystalline synovitis respectively (Ryumachi Gaku, compiled by Hirohata Kazushi et al., Dobun Shoin, 1989). However, such a pharmacotherapy is a symptomatic therapy, and it has hardly inhibited the destruction of joints sufficiently.

In the therapy of rheumatoid arthritis, the selection of the positive therapy to prevent the destruction of joints as much as possible is currently being accepted. The point of this therapy is that a disease is diagnosed as rheumatoid arthritis at the earliest possible stage and antirheumatic drugs such as methotrexate are properly selected. However, sufficient diagnosis has not yet been provided.

A fibroblast growth factor (hereinafter abbreviated as FGF), one of various growth factors existing in vivo, has been known as a heparin-binding growth factor that affects vascular endothelial cells. Further, the FGF family involves 19 types or more, and FGF-2 (basic FGF), FGF-1 (acidic FGF) and the like have been long known. As an FGF receptor, seven types have been to date found, and encode a tyrosine kinase in the intracellular region.

FGF-8 is a factor isolated from a culture supernatant of mouse breast cancer cell line SC-3 (Nakamura N. et al., J. Steroid Biochem., 27, 459-464, 1987) showing sex hormone-dependent growth as an androgen-induced growth factor (AIGF). It is a growth factor which is inductively produced by androgen stimulation and enhances the growth of SC-3 cells in an autocrine manner (Tanaka A. et al., Proc. Natl. Acad. Sci. USA, 89, 8928-8932, 1992). It is reported that FGF-8 accelerates the growth of cells of prostate cancer or fibroblasts (Tanaka A. et al., FEBS Lett., 363, 226-230, 1995). It is reported that FGF-8 bound to three receptors, FGF receptor-2IIIc, FGF receptor-3IIIc and FGF receptor-4 (Ornitz D. M. et al., J. Biol. Chem., 271, 15292-15297, 1996). Moreover, binding to membrane type heparan sulfate proteoglycan such as syndecan is required for the function of FGF. Binding to heparan sulfate

is necessary to the stable and local accumulation of FGF. In the situation of tissue remodelingion such as inflammation, it is considered that heparan sulfate is degraded to liberate FGF from extracellular matrix to exhibit its activity. A strong angiogenesis factor such as FGF-2 is comprised in cartilages (Satoh H. et al., J. Biol. Chem., 273, 12307-12315, 1998). In the arthritis, synovial cells, chondrocytes and inflammatory cells invaded synthesize FGF-1 or FGF-2 at an extremely high level (Sano H. et al., J. Cell Biol., 110, 1417-1426, 1990, Remmers E. F., Growth factors, 2, 179-188, 1990), and the FGF-2 concentration in a synovial fluid of rheumatic patients correlates with arthritis (Manabe N. et al., Rheumatology, 38, 714-720, 1999). FGF-2 is involved in osteophyte formation in osteoarthritis (Uchino M. et al., Clin. Orthop., 377, 119-125, 2000). These reports prove that FGF-1 or FGF-2 is involved in arthritis.

In the report using FGF-8 knockout mice, FGF-8 expressed at the stage of the development of joints (Haraguchi R. et al., Development, 127, 2471-2479, 2000; Lewandoski M. et al., Nat. Genet., 26, 460-463, 2000). Nevertheless, it is unknown that FGF-8 is involved in arthritis.

[Problems to be Solved by the Invention]

It is an object of the invention to provide an agent for preventing or treating arthritis, an agent for inhibiting joint destruction, an agent for cartilage protection, an agent for inhibiting the growth of synovial membrane and a diagnostic agent for arthritis, as well as a diagnostic method for arthritis.

[Means for Solving the Problems]

The invention provides the following (1) to (51).

(1) An agent for preventing or treating arthritis, comprising, as an active ingredient, an antibody which specifically binds to FGF-8 to inhibit activity of FGF-8.

(2) The agent according to (1), wherein the antibody which specifically binds to FGF-8 to inhibit activity of FGF-8 is a monoclonal antibody.

(3) The agent according to (2), wherein the monoclonal antibody is an antibody selected from an antibody produced by a hybridoma, a humanized antibody and an antibody fragment thereof.
(4) The agent according to (3), wherein the hybridoma is hybridoma KM1334 (FERM BP-5451).
(5) The agent according to (3), wherein the humanized antibody is an antibody selected from a human chimeric antibody and a human complementarity determining region (CDR)-grafted antibody.
(6) The agent according to (5), wherein the human chimeric antibody is an antibody in which an antibody heavy chain variable region (VH) comprises an amino acid sequence represented by SEQ ID NO. 2, and/or an antibody light chain variable region (VL) comprises an amino acid sequence represented by SEQ ID NO. 4.
(7) The agent according to (6), wherein the human chimeric antibody is a human chimeric antibody produced by transformant KM3034 (FERM BP-7836).
(8) The agent according to (5), wherein the human CDR-grafted antibody is an antibody in which CDR1, CDR2 and CDR3 of VH comprise amino acid sequences represented by SEQ ID NOS. 5, 6 and 7 respectively, and/or CDR1, CDR2 and CDR3 of VL comprise amino acid sequences represented by SEQ ID NOS. 8, 9 and 10 respectively.
(9) The agent according to (8), wherein the human CDR-grafted antibody is an antibody in which VH comprises amino acid sequence represented by SEQ ID NO. 15 and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16.
(10) The agent according to (8), wherein the human CDR-grafted antibody is an antibody in which VH comprises an amino acid sequence represented by SEQ ID NO. 15 in which at least one or more amino acid residue selected from Lys at position 12, Lys at position 13, Ala at position 40, Pro at position 41, Met at position 48, Val at position 68, Ile at position 70, Thr at position 74, Thr at position 76, Glu at position 82, Ser at position 84, Arg at position 87 and Tyr at position 95

is replaced with another amino acid residue, and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16 in which at least one or more amino acid residue selected from Ile at position 2, Val at position 3, Thr at position 14, Pro at position 15, Gln at position 50, Leu at position 51 and Tyr at position 92 is replaced with another amino acid residue.

(11) The agent according to (3), wherein the antibody fragment is an antibody fragment selected from Fab, Fab', $F(ab')_2$, a single chain antibody (scFv), a dimerized variable region (V region) fragment (diabody), a disulfide-stabilized V region fragment (dsFv) and a CDR-containing peptide.

(12) A diagnostic agent of arthritis comprising an antibody which specifically binds to FGF-8 as an active ingredient.

(13) The diagnostic agent according to (12), wherein the antibody which specifically binds to FGF-8 is a polyclonal antibody or a monoclonal antibody.

(14) The diagnostic agent according to (13), wherein the monoclonal antibody is an antibody selected from an antibody produced by a hybridoma, a humanized antibody and an antibody fragment thereof.

(15) The diagnostic agent according to (14), wherein the hybridoma is hybridoma KM1334 (FERM BP-5451).

(16) The diagnostic agent according to (14), wherein the humanized antibody is an antibody selected from a human chimeric antibody and a human CDR-grafted antibody.

(17) The diagnostic agent according to (16), wherein the human chimeric antibody is an antibody in which VH comprises amino acid sequence represented by SEQ ID NO. 2 and/or VL comprises an amino acid sequence represented by SEQ ID NO. 4.

(18) The diagnostic agent according to (17), wherein the human chimeric antibody is a human chimeric antibody produced by transformant KM3034 (FERM BP-7836).

(19) The diagnostic agent according to (16), wherein the human CDR-grafted antibody is an antibody in which CDR1, CDR2 and CDR3 of VH comprise amino acid sequences represented by SEQ ID NOS. 5, 6 and 7 respectively, and/or CDR1, CDR2 and CDR3

of VL comprise amino acid sequences represented by SEQ ID NOS. 8, 9 and 10 respectively.

(20) The diagnostic agent according to (19), wherein the human CDR-grafted antibody is an antibody in which VH comprises amino acid sequence represented by SEQ ID NO. 15 and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16.

(21) The diagnostic agent according to (19), wherein the human CDR-grafted antibody is an antibody in which VH comprises an amino acid sequence represented by SEQ ID NO. 15 in which at least one or more amino acid residue selected from Lys at position 12, Lys at position 13, Ala at position 40, Pro at position 41, Met at position 48, Val at position 68, Ile at position 70, Thr at position 74, Thr at position 76, Glu at position 82, Ser at position 84, Arg at position 87 and Tyr at position 95 is replaced with another amino acid residue, and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16 in which at least one or more amino acid residue selected from Ile at position 2, Val at position 3, Thr at position 14, Pro at position 15, Gln at position 50, Leu at position 51 and Tyr at position 92 is replaced with another amino acid residue.

(22) The diagnostic agent according to (14), wherein the antibody fragment is an antibody fragment selected from Fab, Fab', $F(ab')_2$, a single chain antibody (scFv), a dimerized V region fragment (diabody), a disulfide-stabilized V region fragment (dsFv) and a CDR-containing peptide.

(23) A diagnostic method for judging arthritis, which comprises detecting and/or determining FGF-8 in a sample using an antibody which specifically binds to FGF-8.

(24) The diagnostic method according to (23), wherein the antibody which specifically binds to FGF-8 is a polyclonal antibody or a monoclonal antibody.

(25) The diagnostic method according to (24), wherein the monoclonal antibody is an antibody selected from an antibody produced by a hybridoma, a humanized antibody and an antibody fragment thereof.

(26) The diagnostic method according to (25), wherein the

hybridoma is hybridoma KM1334 (FERM BP-5451).

(27) The diagnostic method according to (25), wherein the humanized antibody is an antibody selected from a human chimeric antibody and a human CDR-grafted antibody.

(28) The diagnostic method according to (27), wherein the human chimeric antibody is an antibody in which VH comprises amino acid sequence represented by SEQ ID NO. 2 and/or VL comprises an amino acid sequence represented by SEQ ID NO. 4.

(29) The diagnostic method according to (28), wherein the human chimeric antibody is a human chimeric antibody produced by transformant KM3034 (FERM BP-7836).

(30) The diagnostic method according to (27), wherein the human CDR-grafted antibody is an antibody in which CDR1, CDR2 and CDR3 of VH comprise amino acid sequences represented by SEQ ID NOS. 5, 6 and 7 respectively, and/or CDR1, CDR2 and CDR3 of VL comprise amino acid sequences represented by SEQ ID NOS. 8, 9 and 10 respectively.

(31) The diagnostic method according to (30), wherein the human CDR-grafted antibody is an antibody in which VH comprises amino acid sequence represented by SEQ ID NO. 15 and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16.

(32) The diagnostic method according to (30), wherein the human CDR-grafted antibody is an antibody in which VH comprises an amino acid sequence represented by SEQ ID NO. 15 in which at least one or more amino acid residue selected from Lys at position 12, Lys at position 13, Ala at position 40, Pro at position 41, Met at position 48, Val at position 68, Ile at position 70, Thr at position 74, Thr at position 76, Glu at position 82, Ser at position 84, Arg at position 87 and Tyr at position 95 is replaced with another amino acid residue, and/or VL comprises an amino acid sequence represented by SEQ ID NO. 16 in which at least one or more amino acid residue selected from Ile at position 2, Val at position 3, Thr at position 14, Pro at position 15, Gln at position 50, Leu at position 51 and Tyr at position 92 is replaced with another amino acid residue.

(33) The diagnostic method according to (25), wherein the

antibody fragment is an antibody fragment selected from Fab, Fab', $F(ab')_2$, a single chain antibody (scFv), a dimerized V region fragment (diabody), a disulfide-stabilized V region fragment (dsFv) and a CDR-containing peptide.

(34) An agent for inhibiting joint destruction inhibitor comprising, as an active ingredient, an antibody which specifically binds to FGF-8 to inhibit activity of FGF-8.

(35) An agent for protecting cartilage comprising, as an active ingredient, an antibody which specifically binds to FGF-8 to inhibit activity of FGF-8.

(36) An agent for inhibiting growth of synovial membrane comprising, as an active ingredient, an antibody which specifically binds to FGF-8 to inhibit activity of FGF-8.

[Modes for Carrying Out the Invention]

The antibody used in the agent for preventing or treating arthritis in the present invention may be any antibody so long as it is an antibody which specifically binds to FGF-8 and inhibit activity of FGF-8 (hereinafter also referred to as anti-FGF-8 neutralizing antibody). Examples include an antibody having neutralizing activity to FGF-8 and a fragment thereof.

The anti-FGF-8 neutralizing antibody used in the agent for preventing or treating arthritis in the present invention can be obtained by selecting an antibody capable of inhibiting activity of FGF-8 from among antibodies which specifically binds to FGF-8 (hereinafter also referred to as FGF-8 antibodies). The activity of FGF-8 may be any of biological activities that FGF-8 possesses. Specific examples thereof can include an activity that promotes growth of mouse breast cancer cell line SC-3 (Nakamura N. et al., J. Steroid Biochem., 27, 459-464, 1987), mouse fibroblast cell line NIH/3T3 (ATCC No: CRL-1658) or human prostate cancer cell line LNCaP (ATCC No: CRL-1740), an activity that promotes the growth of synovial cells and an activity that promotes degradation of extracellular matrix of chondrocytes.

An anti-FGF-8 antibody can be produced by the known method

(Harlow E. and Lane D., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988, hereinafter referred to as Antibodies A Laboratory Manual).

As the anti-FGF-8 neutralizing antibody used in the agent for preventing or treating arthritis of the present invention, a polyclonal antibody and a monoclonal antibody can both be used. A monoclonal antibody is preferably used.

Examples of the monoclonal antibody can include an antibody produced by a hybridoma, a humanized antibody and an antibody fragment thereof.

The anti-FGF-8 neutralizing monoclonal antibody produced by the hybridoma as used in the agent for preventing or treating arthritis in the invention specifically is produced by the following method.

That is, an FGF-8 protein is prepared as an antigen, and plasma cells having specificity for the antigen are induced in an animal immunized with this antigen. The plasma cells are further fused with myeloma cells to prepare hybridomas, and the hybridomas are cultured. Or the hybridoma cells are administered to an animal to cause ascitic canceration in the animal. Antibodies which specifically bind to FGF-8 are separated from the culture solution or the ascitic fluid, and purified. An antibody that inhibits activity of FGF-8 is selected from among the resulting antibodies. The anti-FGF-8 neutralizing monoclonal antibody includes monoclonal antibody KM1334 produced by hybridoma KM1334 (FERM BP-5451) belonging to mouse IgG1 subclass as described in Japanese published unexamined application No. 271391/97.

The humanized antibody used in the agent for preventing or treating arthritis of the present invention includes the foregoing anti-FGF-8 neutralizing monoclonal antibody which is modified by a gene recombination technology. The antibody having low antigenicity and prolonged blood half-life is preferably in the preventing or treating agent.

The humanized antibody used in the agent for preventing or treating arthritis of the present invention includes a human

chimeric antibody and a human complementary determining region (hereinafter abbreviated as CDR)-grafted antibody.

The human chimeric antibody means an antibody comprising the antibody heavy-chain variable region (the variable region is hereinafter referred to as V region and the heavy-chain variable region as VH) and light-chain V region (hereinafter referred to as VL) of a non-human animal, and the human antibody heavy-chain constant region (the constant region is hereinafter referred to as C region and the heavy-chain constant region as CH) and the human antibody light-chain C region (hereinafter referred to as CL). As the non-human animal, any of animals from which a hybridoma can be prepared, such as mice, rats, hamsters and rabbits, can be used.

The human chimeric antibody used in the agent for preventing or treating arthritis of the present invention can be prepared by obtaining DNAs encoding VH and VL from cDNAs encoding H chain and L chain of the antibody obtained from the hybridoma producing the anti-FGF-8 neutralizing monoclonal antibody, individually inserting the DNAs into a vector for expression in animal cells carrying DNAs encoding human antibody CH and CL to construct a human chimeric antibody expression vector and introducing the vector into the animal cell for expression.

As CH of the human chimeric antibodies, any CH of antibodies belonging to human immunoglobulin (hIg) may be used. CH of antibodies belonging to hIgG class is preferable, and any of subclasses such as γ1, γ2, γ3 and γ4 belonging to hIgG class may be used. As CL of the human chimeric antibodies, any CL of antibodies belonging to hIg, for example, κ class or λ class, may be used.

The human chimeric antibody which specifically binds to FGF-8 to inhibit activity of FGF-8 (hereinafter also referred to as an anti-FGF-8 neutralizing chimeric antibody) includes a human chimeric antibody in which CDR1, CDR2 and CDR3 of VH comprise amino acid sequences represented by SEQ ID NOS. 5, 6 and 7, and/or CDR1, CDR2 and CDR3 of VL comprise amino acid

sequences represented by SEQ ID NOS. 8, 9 and 10, and preferably includes the human chimeric antibody in which VH comprises amino acid sequences represented by SEQ ID NO. 2 and/or VL comprises amino acid sequences represented by SEQ ID NO. 4. Examples include human chimeric antibody KM3034 wherein VH of the antibody consists of the amino acid sequence represented by SEQ ID NO: 4, CH of the antibody consists of an amino acid sequence of the human γ1 subclass, VL of the antibody consists of the amino acid sequence represented by SEQ ID NO: 4 and CL of the andibody consists of an amino acid sequence of the human κ class.

Transformant KM3034 which produces human chimeric antibody KM3034 was deposited as FERM BP-7836 in International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukubashishi, Ibaraki, Japan) on December 26, 2001.

The human CDR-grafted antibody means an antibody prepared by replacing CDRs of VH and VL of an antibody of a non-human animal with CDR sequences of an antibody of a non-human animal respectively in a human antibody.

The human CDR-grafted antibody used in the agent for preventing or treating arthritis of the present invention can be prepared by constructing cDNAs encoding V regions in which CDR sequences of VH and VL of any human antibody are replaced with CDR sequences of VH and VL of an anti-FGF-8 neutralizing antibody of a non-human animal respectively, individually inserting them in a vector for expression in animal cells carrying genes encoding human antibody CH and human antibody CL to construct a human CDR-grafted antibody expression vector and introducing the vector into the animal cell for expression.

As CH of the human CDR-grafted antibodies, any CH of antibodies belonging to hIg may be used. CH of antibodies belonging to hIgG class is preferable, and any of subclasses such as γ1, γ2, γ3 and γ4 belonging to hIgG class may be used. As CL of the human CDR-grafted antibodies, any CL of antibodies belonging to hIg, for example, κ class or λ class, may be used.

The human CDR-grafted antibody which specifically binds

to FGF-8 to inhibit activity of FGF-8 (hereinafter also referred to as an anti-FGF-8 neutralizing CDR-grafted antibody) includes a human CDR-grafted antibody in which CDR1, CDR2 and CDR3 of VH of the antibody comprise amino acid sequences represented by SEQ ID NOS. 5, 6 and 7, respectively, and/or CDR1, CDR2 and CDR3 of VL of the antibody comprise amino acid sequences represented by SEQ ID NOS. 8, 9 and 10, respectively and preferably includes a human CDR-grafted antibody in which VH of the antibody comprises amino acid sequences represented by SEQ ID NO. 15 and/or VL of the antibody comprises amino acid sequences represented by SEQ ID NO. 15. More preferable examples include a human CDR-grafted antibody in which VH comprises an amino acid sequence represented by SEQ ID NO. 15 in which at least one or more amino acid residue selected from Lys at position 12, Lys at position 13, Ala at position 40, Pro at position 41, Met at position 48, Val at position 68, Ile at position 70, Thr at position 74, Thr at position 76, Glu at position 82, Ser at position 84, Arg at position 87 and Tyr at position 95 is replaced with another amino acid residue, and/or VL of the antibody comprises an amino acid sequence represented by SEQ ID NO. 16 in which at least one or more amino acid residue selected from Ile at position 2, Val at position 3, Thr at position 14, Pro at position 15, Gln at position 50, Leu at position 51 and Tyr at position 92 is replaced with another amino acid residue.

The anti-FGF-8 neutralizing antibody used in the agent for preventing or treating arthritis of the present invention includes also an antibody fragment. The antibody fragment includes Fab (abbreviated for fragment of antigen binding), $F(ab')_2$, Fab', a single-chain antibody (single chain Fv; hereinafter reffered to as scFv), a dimerized V region fragment (diabody), a disulfide stabilized antibody (disulfide stabilized Fv; hereinafter referred to as dsFv) and a CDR-containing peptide.

The Fab is a fragment having antigen-binding activity and comprising approximately a half of an N-terminal side of

H chain and the whole L chain, the fragment being obtained by digesting upper peptide moieties of two disulfide bonds that crosslink two H chains in hinge regions of IgG with an enzyme papain having a molecular weight of approximately 50,000.

The $F(ab')_2$ is an antibody fragment obtained by treating lower moieties of two disulfide bonds in hinge regions of IgG with proteinase pepsin (cleaving in 234th amino acid residue of H chain) in which Fab is slightly larger than that bound through disulfide bonds in hinge regions having a molecular weight of approximately 100,000 and having antigen-binding activity.

The Fab' is a fragment obtained by cleaving disulfide bonds between hinges of $F(ab')_2$ having a molecular weight of approximately 50,000 and antigen-binding activity.

The scFv is a VH-P-VL or VL-P-VH polypeptide in which one VH and one VL are linked with an appropriate peptide linker (hereinafter referred to as P). As VH and VL comprised in scFv used in an agent for preventing or treating arthritis in the invention, any VH and VL of anti-FGF-8 neutralizing monoclonal antibodies can be used.

The diabody is an antibody fragment in which scFvs that have the same or different antigen-binding specificities form a dimer, and this is an antibody fragment having divalent antigen-binding activity to the same antigen or antigen-binding activities specific to different antigens respectively.

The dsFv is a fragment in which polypeptides with one amino acid residue of VH and one amino acid residue of VL replaced with cysteine residues are bound through a disulfide bond. The amino acid residue replaced with the cysteine residue can be selected by estimating a three-dimensional structure of an antibody according to the method indicated by Reiter et al. (Reiter Y. et al., Protein Eng., 7, 697-704, 1994). As VH or VL comprised in dsFv used in the agent for preventing or treating arthritis of the present invention, any VH and VL of anti-FGF-8 neutralizing monoclonal antibodies can be used.

The CDR-containing peptide used in the agent for

preventing or treating arthritis of the present invention comprises at least one or more region of CDRs of VH and VL of the anti-FGF-8 neutralizing antibody. The peptide containing plural CDRs can be produced by binding them either directly or through an appropriate peptide linker.

A specific process for producing the anti-FGF-8 neutralizing antibody used of the present invention, a method for evaluating activity thereof, the agent for preventing or treating arthritis comprising the antibody, the diagnostic agent of arthritis comprising the anti-FGF-8 antibody and the method for diagnosing arthritis using the anti-FGF-8 antibody are described below.

1. Process for producing the anti-FGF-8 neutralizing antibody (polyclonal antibody, monoclonal antibody)

(1) Preparation of an antigen

Examples of an antigen necessary for producing the anti-FGF-8 neutralizing antibody include a cell producing FGF-8 or its cell fraction, an FGF-8 protein, a partial fragment of the protein, a peptide having a partial sequence of an amino acid sequence of the protein, and the like.

The FGF-8 protein and the partial fragment of the protein can be produced as such or as a fusion protein intracellularly or in a culture supernatant by constructing a recombinant vector in which a full-length or partial fragment cDNA encoding FGF-8 (Tanaka A. et al., Proc. Natl. Acad. Sci. USA, 89, 8928-8932, 1992, Tanaka A. et al., FEBS Lett., 363, 226-230, 1996) is inserted downstream of a promoter of an appropriate vector, introducing the recombinant vector into a host cell to obtain an FGF-8 expression cell and culturing the cell in an appropriate medium. The peptide having the partial sequence of the FGF-8 protein can be prepared using a peptide synthesizer.

The full-length or partial fragment cDNA encoding FGF-8 can be prepared by a polymerase chain reaction [hereinafter referred to as PCR; Sambrook J. et al., Molecular Cloning 3rd edition, Cold Spring Harbor Laboratory, 2001 (hereinafter

referred to as "Molecular Cloning 3rd edition"), Ausubel F. M. et al., Current Protocols in Molecular Biology, John Wiley & Sons, 1987-2001 (hereinafter referred to as Current Protocols in Molecular Biology) using a cDNA prepares from cells expressing FGF-8, such as SC-3, as a template.

As a host, any of hosts may be used so long as a desired gene can be expressed therein, examples thereof being bacteria, yeasts, animal cells, insect cells and the like. Examples of bacteria include bacteria belonging to the genus Escherichia and the genus Bacillus, such as Escherichia coli and Bacillus subtilis. Examples of yeasts include Saccharomyces cerevisiae and Schizosaccharomyces pombe. Examples of animal cells include a Namalwa cell being a human cell, a COS cell being a monkey cell, a CHO cell being a cell of Chinese hamster and the like. Examples of insect cells include Sf9 and Sf21 (manufactured by Pharmingen), High Five (manufactured by Invitrogen) and the like.

As the vector into which to introduce a full-length or partial fragment cDNA encoding FGF-8, any of vectors can be used, so long as the DNA can be incorporated therein and expressed in a host cell.

When bacterium such as Escherichia coli is used as a host, the expression vector comprising a promoter, a ribosome-binding sequence, a full-length or partial fragment cDNA encoding FGF-8, a transcription termination sequence and, as required, a promoter controlling sequence is preferable. Examples thereof include commercially available pGEX-2T (manufactured by Amersham Biosciences) and pET17b (manufactured by Novagen).

As a method for introducing a recombinant vector into bacteria, any method can both be used so long as a DNA is introduced into bacteria, for example, the method using calcium ion (Cohen S. N. et al., Proc. Natl. Acad. Sci., USA, 69, 2110-2114, 1972) and the protoplast method (Japanese published unexamined application No. 248394/88).

When yeasts is using as a host, for example, YEp13 (ATCC 37115), YEp24 (ATCC 37051) and YCp50 (ATCC 37419) are used as

the expression vector.

As the method for introducing the recombinant vector into yeasts, any method can be used so long as a DNA is introduced into yeasts. Example includes the electroporation method (Becker D. M. and Guarente L., Methods, Enzymol., 194, 182-187, 1991), the spheroplast method (Hinnen A. et al., Proc. Natl. Acad. Sci. USA, 84, 1929-1933, 1978), the lithium acetate method (Ito H. et al., J. Bacteriol., 153, 163-168, 1983) and the like.

When an animal cell is using as a host, for example, pAGE107 (Japanese published unexamined application No. 22979/91; Miyaji H. et al., Cytotechnology, 3, 133-140, 1990) and pAGE103 (Mizukami T. and Itoh S., J. Biochem., 101, 1307-1310, 1987) are used as the expression vector.

Any promoter can be used, so long as it can be expressed in animal cells can be used. Examples include an IE (immediate early) gene promoter of cytomegalovirus (CMV), a promoter of SV40 or metallothionein, and the like. An enhancer of the IE gene of human CMV may be used along with the promoter.

As a method for introducing a recombinant vector into animal cells, any of methods in which a DNA is introduced into animal cells, such as the electroporation method (Miyaji H. et al., Cytotechnology, 3, 133-140, 1990), the calcium phosphate method (Japanese published unexamined application No. 227075/90) and the lipofection method (Felgner P. L. et al., Proc. Natl. Acad. Sci. USA, 84, 7413-7417, 1987), can be used.

When the insect cells are used as a host, a protein can be expressed by the method described in, for example, Current Protocols in Molecular Biology, O' Reilly et al., Baculovirus Expression Vectors: A Laboratory Manual, Oxford University Press, 1994 or the like. That is, the following recombinant gene introduction vector and baculovirus are co-introduced into insect cells to obtain a recombinant virus in the insect cells culture supernatant, and the insect cells are infected with the recombinant virus to obtain protein expression insect cells.

As the gene introduction vector, for example, pVL1392 and pVL1393 (both manufactured by Pharmingen), pBlueBac4.5

(manufactured by Invitrogen) and the like are used.

As the baculovirus, for example, Autographa californica nuclear polyhedrosis virus, a virus with which insects of the family Barathra are infected, is used.

As the method for co-introducing the recombinant gene introduction vector and the baculovirus for preparation of the recombinant virus, for example, the calcium phosphate method (Japanese published unexamined application No. 227075/90) and the lipofection method (Felgner P. L. et al., Proc. Natl. Acad. Sci. USA, 84, 7413-7417, 1987) and the like are used.

A protein may be produced by preparing a recombinant baculovirus using Baculo Gold Starter Kit manufactured by Pharmingen or the like and then infecting insect cells such as Sf9, Sf21, High Five and the like as mentioned above with the recombinant virus (Bio/Technology, 6, 47, 1988).

As the method for expressing the gene, the secretory production, the fusion protein expression and the like, besides the intracellular expression of the FGF-8 protein alone, have been developed, and any of these methods can be used. For example, the expression can be performed according to the method described in Molecular Cloning 3rd edition.

The thus-obtained transformant is cultured in a medium to form and accumulate the FGF-8 protein in the culture, and the FGF-8 protein is extracted from the culture, whereby the full length or the partial fragment of the FGF-8 protein can be produced as such or as a fusion protein.

The method for culturing the transformant in a medium is carried out according to an ordinary method used in culturing a host.

As the medium in for culturing the transformant obtained by using microorganisms such as Escherichia coli or yeast as a host, either a natural medium or a synthetic medium can be used, so long as it comprises carbon sources, nitrogen sources, inorganic salts and the like which can be assimilable by the microorganisms and culturing the transformants efficiently can be carried out (Molecular Cloning 3rd edition). The culturing

is usually carried out under aerobic conditions such as shaking culture or submerged aeration-agitation culture at 15 to 40°C for 16 to 96 hours. During the culturing, the pH is maintained at from 3.0 to 9.0. The pH is adjusted with an inorganic or organic acid, an alkaline solution, urea, calcium carbonate, ammonia or the like. If necessary, antibiotics such as ampicillin and tetracycline can be added to the medium during the culturing.

As the medium for culturing the transformant obtained by using animal cells as a host, RPMI 1640 medium, Eagle's MEM medium, these mediums containing fetal bovine serum (hereinafter abbreviated as FBS) and the like which are generally used are available. The culturing is usually carried out in the presence of 5% CO_2 at 35 to 37°C for 3 to 7 days. If necessary, antibiotics such as kanamycin and penicillin can be added to the medium during the culturing.

As the medium for culture the transformant obtained by using insect cells as a host, TNM-FH medium (manufactured by Pharmingen), Sf900IISFM (manufactured by Invitrogen), EX-CELL400 and EX-CELL405 (both manufactured by JRH Biosciences) and the like which are generally used are available. The culturing is carries out at 25 to 30°C for 1 to 4 days. If necessary, antibiotics such as gentamicin can be added to the medium during the culturing.

In the foregoing culturing of the animal cells and the insect cells, it is preferable, if possible, to use a serum-free medium for facilitating purification of the full length or the partial fragment of FGF-8 as such or as a fusion protein.

When the full length or the partial fragment of FGF-8 is accumulated inside host cells as such or as a fusion protein, the cells are centrifuged after completion of the culturing, suspended in an aqueous buffer, and the ultrasonic disruption method, the French press method or the like. The protein is recovered from the supernatant obtained by centrifugation.

Also, when insolved body is formed intracellularly, aprotein can be made into three-dimensional structure by

diluting or dialyzing the solubilized solution in at such a concentration of the protein denaturing agent that does not denature protein or without the protein denaturing agent, after solibilized with a protein denaturing agent.

When the full length or the partial fragment of the FGF-8 protein or the fusion protein of these proteins is secreted extracellularly, the expressed protein can be recovered from the culture supernatant.

The isolation and purification can be carried out by using separation procedures such as solvent extraction, fractional precipitation by an organic solvent, salting-out, dialysis, centrifugation, ultrafiltration, ion exchange chromatography, gel filtration chromatography, hydrophobic chromatography, affinity chromatography, reversed phase chromatography, crystallization and electrophoresis alone or in combination.

The peptide having a partial sequence of the amino acid sequence of FGF-8 can be produced by the chemical synthesis methods such as the Fmoc (fluorenylmethyloxycarbonyl) method and the tBoc (t-butyloxycarbonyl) method. It can also be produced using peptide synthesizers of Advanced ChemTech, Applied Biosystems, Protein Technologies, Shimadzu Corporation and the like.

(2) Immunization of animals and preparation of antibody-producing cells

Animals are immunized using the above-obtained protein as an antigen. With respect to the immunization method, the antigen may directly be administered to animals subcutaneously, intravenously or intraperitoneally. It is preferable to administer the antigen combined with a carrier protein having high immunogenicity or administer the antigen along with an appropriate adjuvant.

Examples of the carrier protein include Keyhole limpet hemocyanin, bovine serum albumin, bovine thyroglobulin and the like. Examples of the adjuvant include Complete Freund's Adjuvant, aluminum hydroxide gel, pertussis bacteria vaccine and the like.

Examples of immunized animals include non-human mammals such as rabbits, goats, mice, rats and hamsters.

The antigen is administered, after the first administration, 3 to 10 times every 1 to 2 weeks. The dose of the antigen is preferably 50 to 100 μg per animal. The blood sample is collected from the venous plexus of the fundus of the eye or the tail vein of the immunized animal 3 to 7 days after each administration. The specific binding ability with the antigen of the serum is confirmed by enzyme immunoassay [Koso Men-eki Sokuteiho, 3rd edition, Igaku Shoin, 1987, Antibodies A Laboratory Manual (Chapter 14), Goding J. W., Monoclonal Antibodies: Principles and Practice, Academic Press, 1996 (hereinafter abbreviated as Monoclonal Antibodies] or the like as described below.

The enzyme immunoassay can be performed as follows.

An antigen protein or cells with an antigen protein expressed are coated on a plate, and reacted with a serum collected from immunized animals as a first antibody. After the reaction of the first antibody, the plate is washed, and a second antibody is added thereto. After the reaction, a detection reaction corresponding to a substance labeled with the second antibody is carried out, and an antibody titer is measured.

The second antibody is an antibody capable of recognizing the first antibody, which is labeled with an enzyme such as peroxidase or biotin. Specifically, when a mouse is used as the immunized animal, an antibody capable of recognizing mouse immunoglobulin is used as the second antibody.

Non-human mammals in which the serum shows a sufficiently antibody titer are used as a supply source of antibody-producing cells.

After 3 to 7 days from the final administration of the antigen, lymphocytes are extracted from the immunized animals to fuse them with myeloma cells, according to the known method (Antibodies A Laboratory Manual).

The polyclonal antibody can be prepared by separating

and purifying the serum. Whether the polyclonal antibody has the neutralizing activity to inhibit the activity of FGF-8 can be examined by cell growth inhibition assay described in 1. (4) below.

The monoclonal antibody can be prepared by fusing the antibody-producing cells with the myeloma cells derived from non-human mammals to produce hybridomas, culturing the hybridomas or administering the hybridomas to animals to from ascitic tumor of the cells and separating and purifying the culture solution or the ascitic fluid.

The antibody-producing cells can be extracted from spleen cells, lymph nodes, peripheral bloods and the like of the antigen-administered non-human mammals.

(3) Preparation of myeloma cells

As the myeloma cells, any of myeloma cells capable of growth in vitro, such as 8-azaguanine-resistant mouse (derived from BALB/c) myeloma cell lines P3-X63Ag8-U1 (Kohler G and Milstein C, Eur. J. Immunol., 6, 511-519, 1976), SP2/0-Ag14 (Shulman M. et al., Nature, 276, 269-270, 1978), P3-X63-Ag8653 (Kearney J. F. et al., J. Immunol., 123, 1548-1550, 1979) and P3-X63-Ag8 (Kohler G and Milstein C, Nature, 256, 495-497, 1975) which are established cell lines obtained from a mouse, can be used. With respect to the culturing and the subculturing of these cell lines, the number of cells up to at least 2 x 10^7 cells or more is secured until the cell fusion according to the known method (Antibodies A Laboratory Manual).

(4) Cell fusion and selection of a monoclonal antibody

The above-obtained antibody-producing cells and myeloma cells are washed, and a cell aggregating medium such as polyethylene glycol-1000 (PEG-1000) is added thereto to fuse the cells. The fused cells are suspended in a medium. The cells are washed using MEM medium, PBS (1.83 g/L Na_2HPO_4, 0.21 g/L KH_2PO_4, 7.65 g/L NaCl, 1 liter of distilled water, pH 7.2) or the like. As the medium in which to suspend the fused cells, HAT medium [medium obtained by adding 10^{-4} mol/L hypoxanthine, 1.5×10^{-5} mol/L thymidine and 4×10^{-7} mol/L aminopterin to a normal

medium (RPMI 1640 medium containing 1.5 mol/L glutamine, 5×10^{-5} mol /L 2-mercaptoethanol, 10 g/mL gentamicin and 10% FBS) is used to selectively obtain desired fused cells alone.

After the culturing, a part of the culture supernatant is sampled, and reacted with an antigen protein by the following enzyme immunoassay to select a sample that is not reacted with a non-antigen protein. Subsequently, cloning is performed by the limiting dilution method, and the cell in which a high antibody titer is stably measured by the enzyme immunoassay is selected as a monoclonal antibody-producing hybridoma cell line which specifically binds to FGF-8.

The enzyme immunoassay is performed as described in 1. (2) except that the hybridoma culture supernatant or a purified antibody obtained by a method to be described later is used as the first antibody.

The specific binding between the monoclonal antibody and FGF-8 can also be evaluated by the surface plasmon resonance (Karlsson R. et al., J. Immunol. Methods, 145, 229-240, 1991).

Specific examples of the anti-FGF-8 monoclonal antibody include monoclonal antibody KM1334 produced by hybridoma KM1334 (FERM BP-5451) belonging to mouse IgG1 subclass as described in Japanese published unexamined application No. 271391/97.

Whether the anti-FGF-8 monoclonal antibody produced by the above-selected hybridoma can inhibit the activity of FGF-8 is examined by growth inhibition assay using, as a target cell, mouse breast cancer cell line SC-3 (Nakamura N. et al., J. Steroid Biochem., 27, 459-464, 1987), mouse fibroblast NIH/3T3 (ATCC No. CRL-1658) or human prostatic cancer cell line LNCaP (ATCC No: CRL-1740). In the method, when the target cell is cultured in a medium containing FGF-8 (from 1 to 100 ng/mL) or testosterone, the culture supernatant or the anti-FGF-8 monoclonal antibody purified according to the method described in 2. (5) below is stepwise diluted to a final concentration of 0.001 to 100 μg/mL, and added to the medium. After the culturing for 24 to 72 hours, the number of living cells is measured using an MTT [3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl-2H-tetrazoli

um bromide] solution, a cell counting kit, WST-1 Kit or the like. When the number of living cells is decreased dependently on the concentration of the anti-FGF-8 monoclonal antibody in comparison to the case of not adding the anti-FGF-8 monoclonal antibody, it can be confirmed that the anti-FGF-8 monoclonal antibody is an anti-FGF-8 neutralizing antibody that inhibits the activity of FGF-8.

The activity of inhibiting the binding of FGF-8 to the receptor on the cell surface by the anti-FGF-8 monoclonal antibody can be measured by the Bolton-Hunter method (Bolton A. E. and Hunter W. M., Biochem. J., 133, 529-539, 1973) or the like using a system of measuring the binding of ^{125}I-labeled FGF-8 to the foregoing cell line.

The foregoing monoclonal antibody KM1334 is an anti-FGF-8 neutralizing antibody having the activity of inhibiting the FGF-8 activity, and it is preferable as the agent for preventing or treating arthritis.

(5) Preparation of a monoclonal antibody

Monoclonal antibody-producing hybridoma cells are intraperitoneally administered to 8- to 10-week-old mice or nude mice fed for 2 weeks by intraperitoneally administering 0.5 mL of a culture solution formed by culturing hybridoma cells or Pristane (2,6,10,14-tetramethylpentadecane) to cause ascitic canceration, and the monoclonal antibody can be prepared by being separated and purified from the resulting ascitic fluid.

As the method for separating and purifying the monoclonal antibody, centrifugation, salting-out with 40 to 50% saturated ammonium sulfate, method of caprylic acid precipitation, chromatographies using DEAE-Sepharose column, anion exchange column, protein A- or G-column and gel filtration column, and the like are used alone or in combination. The purified monoclonal antibody can be obtained by recovering the IgG or IgM fraction by this method.

The subclass of the purified monoclonal antibody can be determined using a monoclonal antibody typing kit or the like. The amount of the protein can be calculated by the Lowry method

or absorbance at 280 nm.

The subclass of the antibody is an isotype of the class. Examples thereof include IgG1, IgG2a, IgG2b and IgG3 in mouse, and IgG1, IgG2, IgG3 and IgG4 in humans. Especially mouse IgG1 and IgG2a types and human IgG1 type have complement-dependent cytotoxic activity (hereinafter referred to as CDC activity) and antibody-dependent cytotoxic activity (hereinafter referred to as ADCC activity), and are useful in the therapeutic application.

2. Process for producing an anti-FGF-8 neutralizing humanized antibody

(1) Construction of a vector for expression of humanized antibody

A vector for expression of humanized antibody necessary for producing a humanized antibody from an antibody of a non-human animal is constructed. The vector for expression of humanized antibody is a vector for expression in animal cells having inserted therein genes encoding CH and CL which are C regions of a human antibody, and can be constructed by inserting genes encoding CH and CL of a human antibody in a vector for expression in animal cells.

The C regions of the human antibody can be CH and CL of any human antibody. Examples thereof include CH of γ1 subclass, CH of γ4 subclass and CL of κ class of a human antibody, and the like. As DNAs encoding CH and CL of a human antibody, chromosomal DNAs comprising exons and introns can be used, and cDNAs are also available. As the vector for expression in animal cells, any of vectors can be used so long as genes encoding C regions of a human antibody can be inserted and expressed therein.

Examples thereof include pAGE107 (Japanese published unexamined application No. 22979/91; Miyaji H. et al., Cytotechnology, 3, 133-140, 1990), pAGE103 (Mizukami T. and Itoh S., J. Biochem., 101, 1307-1310, 1987), pHSG274 (Brady G. et al., Gene, 27, 223-232, 1984), pKCR (O'Hare K. et al., Proc. Natl. Acad. Sci. USA., 78, 1527-1531, 1981), pSG1βd2-4

(Miyaji H. et al., Cytotechnology, 4, 173-180, 1990) and the like. Examples of a promoter and an enhancer used in the vector for expression in animal cell include initial promoter and enhancer of SV40 (Mizukami T. and Itoh S., J. Biochem., 101, 1307-1310, 1987), LTR promoter and enhancer of Moloney mouse leukemia virus (Kuwana Y. et al., Biochem. Biophys. Res. Commun., 149, 960-968, 1987) and a promoter (Mason J. O. et al., Cell, 41, 479-487, 1985) and an enhancer (Gillies S. D. et al., Cell, 33, 717-728, 1983) of immunoglobulin H chain, and the like.

As the vector for expression of humanized antibody, a type in which antibody H chain and L chain are present in different vectors or a type in which they are present in one and the same vector (tandem-type) can both be used. A tandem-type vector for expression of humanized antibody is preferable in view of ease of construction of the humanized antibody expression vector, ease of introduction into animal cells and a balance of the amount of the expressed antibody H chain and L chain in animal cells (Shitara K. et al., J. Immunol. Methods, 167, 271-278, 1994). Examples of the tandem-type vector for expression of humanized antibody include pKANTEX93 (WO 97/10354), pEE18 (Bentley K. J. et al, Hybridoma, 17, 559-567, 1998) and the like.

The constructed vector for expression of humanized antibody can be used in the expression of a human chimeric antibody and a human CDR-grafted antibody in animal cells.

(2) Preparation of cDNAs encoding VH and VL of an anti-FGF-8 neutralizing antibody of a non-human animal

cDNAs encoding VH and VL of an anti-FGF-8 neutralizing antibody of a non-human animal, for example, a mouse anti-FGF-8 neutralizing monoclonal antibody are obtained as follows.

mRNA are extracted from cells producing a mouse anti-FGF-8 neutralizing monoclonal antibody, for example, hybridomas producing a mouse FGF-8 neutralizing antibody, and cDNAs are synthesized. The synthesized cDNAs are inserted into vectors such as phages or plasmids to produce a cDNA library. From this library, a recombinant phage or a recombinant plasmid having

a cDNA encoding VH and a recombinant phage or a recombinant plasmid having a cDNA encoding VL are isolated respectively using a C region moiety or a V region moiety of a mouse antibody as a probe.

The full length nucleotide sequences of VH and VL in the recombinant phage or the recombinant plasmid are determined, and the full length amino acid sequences of VH and VL are estimated from the nucleotide sequences.

As a non-human animal, any of animals capable of producing hybridomas, such as mice, rats, hamsters and rabbits, can be used. The process for preparing total RNAs from hybridomas includes the guanidine thiocyanate-cesium trifluoroacetate method (Okayama H. et al., Methods Enzymol., 154, 3-28, 1987), and the process for preparing mRNAs from total RNAs includes the oligo (dT) immobilization cellulose column method (Molecular Cloning 3rd edition) or the like. Examples of a kit for preparing mRNAs from hybridomas include FastTrack mRNA Isolation Kit (manufactured by Invitrogen), QuickPrep mRNA Purification Kit (manufactured by Amersham Biosciences) and the like.

Examples of a process for synthesizing cDNAs and producing a cDNA library include the usual process (Molecular Cloning 3rd edition; Current Protocols in Molecular Biology) and the process using commercially available kits such as SuperScript Choice System for cDNA Synthesis (manufactured by Invitrogen), ZAP-cDNA Synthesis Kit (manufactured by Stratagene) and TimeSaver cDNA Synthesis Kit (manufactured by Amersham Biosciences).

As a vector in which to incorporate a cDNA synthesized using an mRNA extracted from a hybridoma as a template in producing a cDNA library, any of vectors capable of subcloning the cDNA can be used. Examples thereof include phage and plasmid vectors such as ZAP Express (manufactured by Stratagene), pBluescript II SK(+) (manufactured by Stratagene), λZAPII (manufactured by Stratagene), λgt10 (manufactured by Stratagene), λgt11 (manufactured by Stratagene), Lambda

BlueMid (manufactured by Clontech), λExCell (manufactured by Amersham Biosciences), pcD2 (Okayama H. and Berg P., Mol. Cell. Biol., 3, 280-289, 1983) and pUC18 (Yanisch-Perron C. et al., Gene 33, 103-119, 1985).

As Escherichia coli in which to introduce the cDNA library constructed by the phage or plasmid vector, any Escherichia coli capable of introducing, expressing and maintaining the cDNA library can be used. Examples thereof include XL1-Blue MRF' (manufactured by Stratagene), C600 (Appleyard R. K. Genetics, 39, 440-452, 1954), Y1088 (Young R. A. and Davis R., Science, 222, 778-782, 1983), Y1090 (Young R. A. and Davis R., Science, 222, 778-782, 1983), NM522 (Gough J. A. and Murray N. E., J. Mol. Biol., 166, 1-19, 1983), K802 (Wood W. B., J. Mol. Biol., 16, 118-133, 1966), JM105 (Yanisch-Perron C. et al., Gene, 33, 103-119, 1985) and the like.

The cDNA clones encoding VH and VL of the anti-FGF-8 neutralizing antibody of the non-human animal can be selected from the cDNA library by the colony hybridization method or the plaque hybridization method using an isotope or fluorescence-labeled probe (Molecular Cloning 3rd edition). Further, cDNAs encoding VH and VL can also be prepared through PCR using prepared primers and using cDNAs or a cDNA library synthesized from mRNAs as a template.

The nucleotide sequences of the cDNAs selected by the foregoing method can be determined by a reaction based on the di-deoxy method (Sanger F. et al., Proc. Natl. Acad. Sci. USA, 74, 5463-5467, 1977) using the cDNAs cloned in an appropriate vector and analysis using a DNA sequencer such as ABI377 (manufactured by Applied Biosystems) or the like.

(3) Analysis of amino acid sequences of VH and VL of an anti-FGF-8 neutralizing antibody of a non-human animal and identification of amino acid sequences of CDRs

The full length amino acid sequences of VH and VL encoded by the cDNAs are estimated from the nucleotide sequences of the cDNAs obtained and determined in 2. (2), and it can be confirmed whether the resulting cDNAs encode the full length

amino acid sequences of VH and VL of an antibody containing a secretory signal sequence in comparison to the full length amino acid sequences of VH and VL of the known antibody (Sequences of Proteins of Immunological Interest, US Dept. Health and Human Services, 1991, hereinafter referred to as "Sequences of Proteins of Immunological Interest"). With respect to the full length amino acid sequences of VH and VL of the antibody containing the secretory signal sequence, it is possible to estimate the length of the secretory signal sequence and the N-terminal amino acid sequence and know subgroups to which they belong in comparison to the full length amino acid sequences of VH and VL of the known antibody (Sequences of Proteins of Immunological Interest).

The novelty of the sequences can be examined by performing search for homology of the full length amino acid sequences of the resulting VH and VL using a homology search program such as BLAST (Altschul S. F. et a., J. Mol. Biol., 215, 403-410, 1990) or the like with respect to any database, for example, SWISS-PROT or PIR-Protein.

VH and VL that form antigen-binding sites of an antibody comprise four framework regions (hereinafter referred to as "FRs") with relatively conserved sequences and three CDRs (CDR1, CDR2, CDR3) with varied sequences linking those (Sequences of Proteins of Immunological Interest). The amino acid sequences of CDRs of VH and VL can be identified in comparison to amino acid sequences of V regions of the known antibody (Sequences of Proteins of Immunological Interest).

(4) Construction of an anti-FGF-8 neutralizing chimeric antibody expression vector

The anti-FGF-8 neutralizing chimeric antibody expression vector can be constructed by inserting cDNAs encoding VH and VL of the anti-FGF-8 neutralizing antibody of the non-human animal uptream of genes encoding CH and CL of the human antibody of the vector for expression of humanized antibody constructed in 2.(1). For example, VH and VL of the anti-FGF-8 neutralizing antibody of the non-human animal are amplified by the PCR method

using a plasmid having cDNAs encoding VH and VL of the antibody as a template and primers on the 5'-terminal side and the 3'-terminal side, the primers comprising recognition sequences of appropriate restriction endonucleases and nucleotide sequences encoding V regions. The respective amplified products are cloned into a plasmid such as pBluescript II SK(-) (manufactured by Stratagene), and the nucleotide sequences are determined by the method described in 2. (2) to obtain a plasmid having the DNA sequences encoding the amino acid sequences of VH and VL of the anti-FGF-8 neutralizing antibody. The cDNAs encoding the amino acid sequences of VH and VL of the anti-FGF-8 neutralizing antibody are isolated from the resulting plasmid, cloned upstream of the genes encoding CH and CL of the human antibody of the vector for expression of humanized antibody described in 2.(1) such that these are expressed in an appropriate from. In this manner, the anti-FGF-8 neutralizing chimeric antibody expression vector can be constructed.

(5) Construction of cDNAs encoding V regions of an anti-FGF-8 neutralizing CDR-grafted antibody

cDNAs encoding VH and VL of the anti-FGF-8 neutralizing CDR-grafted antibody can be constructed as follows. First, amino acid sequences of FRs of VH and VL of a human antibody on which to graft amino acid sequences of CDRs of VH and VL of the anti-FGF-8 neutralizing antibody of the non-human animal are selected. As the amino acid sequences of FRs of VH and VL of the human antibody, any of amino acid sequences derived from the human antibody can be used. Examples thereof include amino acid sequences of VH and VL of the human antibody registered in datanucleotide such as Protein Data Bank and consensus amino acid sequences of subgroups of FRs of VH and VL of the human antibody (Sequences of Proteins of Immunological Interest). Among these, it is preferable to select amino acid sequences having as high a homology to the amino acid sequences of FRs of VH and VL of the anti-FGF-8 neutralizing antibody of the non-human animal as possible, preferably amino acid sequences having a homology thereto by 60% or more for producing the human

CDR-grafted antibody having the sufficiently activity.

Subsequently, the desired amino acid sequences of CDRs of VH and VL of the anti-FGF-8 neutralizing antibody of the non-human animal are grafted on the selected amino acid sequences of FRs of VH and VL of the human antibody to design the amino acid sequences of VH and VL of the anti-FGF-8 neutralizing CDR-grafted antibody. The designed amino acid sequences are converted to nucleotide sequences in consideration of the codon frequency found in the nucleotide sequences of the genes of the antibody (Sequences of Proteins of Immunological Interest) to design the nucleotide sequences encoding the amino acid sequences of VH and VL of the anti-FGF-8 neutralizing CDR-grafted antibody. According to the designed nucleotide sequences, several synthetic DNAs having a length of about 100 nucleotides are synthesized, and PCR is carried out using them. In this case, it is preferable to design six synthetic DNAs of each of VH and VL in view of PCR reaction efficiency and length of DNAs that can be synthesized. Further, DNAs can easily be cloned in the vector for expression of humanized antibody constructed in 2. (1) by introducing recognition sequences of appropriate restriction endonucleases on the 5'-terminals of the synthetic DNAs located at the both ends. After the PCR reaction, the amplified products are cloned in a plasmid vector such as pBluescript SK(-) (manufactured by Stratagene), and the nucleotide sequences are determined by the method described in 2. (2) to obtain the plasmid having the nucleotide sequences encoding the amino acid sequences of VH and VL of the desired anti-FGF-8 neutralizing CDR-grafted antibody.

(6) Modification of amino acid sequences of VH and VL of a human CDR-grafted antibody

It is known that the antigen-binding activity of the desired human CDR-grafted antibody is decreased as compared to the original activity of the antibody of the non-human animal by grafting only CDRs of VH and VL of the antibody of the non-human animal on FRs of VH and VL of the human antibody (Tempest P. R. et al., Bio/technology, 9, 266-271, 1991). With respect

to its cause, some amino acid residues of not only CDRs but also FRs are involved in the antigen-binding activity directly or indirectly in VH and VL of the original antibody of the non-human animal, and these amino acid residues are considered to be changed to another amino acid residues of FRs of VH and VL of the human antibody according to the grafting of CDRs. In order to solve this problem, in the human CDR-grafted antibody, the amino acid residues which are directly involved in the binding to the antigen or the amino acid residues which interact with the amino acid residues of CDRs or maintain the three-dimensional structure of the antigen and are indirectly involved in the binding to the antigen are identified in the amino acid sequences of FRs of VH and VL of the human antibody, and they are replaced with amino acid residues found in the original antibody of the non-human animal to increase the decreased antigen-binding activity (Tempest P. R. et al., Bio/technology, 9, 266-271, 1991). In the production of the human CDR-grafted antibody, the most important point is how efficiently the amino acid resides of FRs involved in the antigen-binding activity are identified. To this end, the construction and the analysis of the three-dimensional structure of the antibody are performed by X-ray crystallography (Bernstein F. C. et al., J. Mol. Biol., 112, 535-542, 1977), computer modeling (Tempest P. R. et al., Protein Engineering, 7, 1501-1507, 1994) or the like. The information of the three-dimensional structure of the antibody obtained by these methods has provided a lot of useful information in the production of the human CDR-grafted antibody. Meanwhile, a process for producing a human CDR-grafted antibody which can be applied to any antibodies has not yet been established. At present, various trial-and-error testings are required in which several types of variants are produced for the respective antibodies and the interrelation of the antigen-binding activities thereof are examined.

The modification of the amino acid residues of FRs of VH and VL of the human antibody can be achieved by performing

PCR method using synthetic DNAs as primers for mutagenesis. With respect to the amplified products after PCR, the nucleotide sequences thereof are determined by the method described in 2.(2) to confirm that the desired modification has been carried out, whereby the vector comprising DNAs with the desired modification introduced (hereinafter referred to as an amino acid sequence-modified vector) is obtained.

The modification of the amino acid sequences in a narrow region is performed by the mutagenesis methods of PCR using mutagenesis primers comprising 20 to 35 bases. Specifically, a sense mutagenesis primer and an antisense mutagenesis primer comprising 20 to 35 bases and comprising DNA sequences encoding amino acid residues after the modification are synthesized, and two-step PCR is performed using a plasmid comprising cDNAs encoding amino acid sequences of VH and VL to be modified as a template. After the final amplified fragment is subcloned into an appropriate vector, its nucleotide sequence is determined to obtain an amino acid sequence-modified vector comprising DNAs with the desired mutagenesis.

(7) Construction of an anti-FGF-8 neutralizing CDR-grafted antibody expression vector

The anti-FGF-8 neutralizing CDR-grafted antibody expression vector can be constructed by cloning cDNAs encoding VH and VL of the anti-FGF-8 neutralizing CDR-grafted antibody constructed in 2.(5) and (6) into upstream of DNAs encoding CH and CL of the human antibody of the vector for expression of humanized antibody described in 2.(1). For example, recognition sequences of appropriate restriction endonucleases are introduced into the 5'-terminals of the synthetic DNAs located at the both ends among the synthetic DNAs used to construct VH and VL of the anti-FGF-8 neutralizing CDR-grafted antibody in 2. (5) and (6), whereby the cloning can be performed such that these are expressed in an appropriate form upstream of DNAs encoding CH and CL of the human antibody of the vector for expression of humanized antibody described in 2.(1).

(8) Transient expression of a humanized antibody and evaluation

of its activity

In order to efficiently evaluate the antigen-binding activity of many types of the humanized antibodies produced, the transient expression of the humanized antibodies can be carried out using the anti-FGF-8 neutralizing chimeric antibody expression vector described in 2. (4), the anti-FGF-8 neutralizing CDR-grafted antibody expression vector described in 2.(7) or modified expression vectors thereof. As the host cell in which to introduce the expression vector, any of host cells capable of expressing the humanized antibody can be used. COS-7 cell (ATCC No: CRL-1651) is generally used owing to its large expression amount (Warr G. W. et al., Methods in Nucleic Acids Research, CRC Press, 283, 1990). Examples of the method for introducing the expression vector into COS-7 cell include the DEAE-dextran method (Warr G. W. et al., Methods in Nucleic Acids Research, CRC press, 283, 1990) and the lipofection method (Felgner P. L. et al., Proc. Natl. Acad. Sci. USA, 84, 7413-7417, 1987).

After the introduction of the expression vector, the expression amount of the humanized antibody in the culture supernatant and the antigen-binding activity thereof can be measured by the enzyme immunoassay, as described in 1. (2), using the culture supernatant as the first antibody and the labeled anti-human immunoglobulin antibody as the second antibody, or the like. Further, whether the neutralizing activity by which to inhibit the FGF-8 activity is retained or not can be confirmed by the cell growth inhibition assay described in 1. (4).

(9) Stable expression of a humanized antibody and evaluation of its activity

A transformant that stably produces the humanized antibody can be obtained by introducing the anti-FGF-8 neutralizing chimeric antibody expression vector described in 2. (4) or the anti-FGF-8 neutralizing CDR-grafted antibody expression vector described in 2. (7) into an appropriate host cell.

The method for introducing the expression vector into the host cell includes the electroporation method (Japanese published unexamined application No. 257891/90; Miyaji H. et al., Cytotechnology, 3, 133-140, 1990).

As the host cell in which to introduce the anti-FGF-8 neutralizing chimeric antibody expression vector or the anti-FGF-8 neutralizing CDR-grafted antibody expression vector, any of host cells capable of expressing the humanized antibody can be used. Examples thereof include mouse SP2/0-Ag14 cell (ATCC No: CRL-1581), mouse P3X63-Ag8.653 cell (ATCC No: CRL-1580), a CHO cell deficient in dihydrofolic acid reductase gene (hereinafter abbreviated as DHFR gene) (Urlaub G. and Chasin L. A., Proc. Natl. Acad. Sci. USA, 77, 4216-4220 1980), rat YB2/3HL.P2.G11.16Ag.20 cell (ATCC No: CRL-1662, hereinafter referred to as YB2/0 cell), and the like.

The transformant that stably produces the humanized antibody after the introduction of the expression vector can be selected by the culturing in an animal cell culture medium containing a compound such as G418 (G418 sulfate; manufactured by Sigma-Aldrich)(Shitara K. et al., J. Immunol. Methods, 167, 271-278, 1994). As the animal cell culture medium, RPMI 1640 medium (manufactured by Nissui Pharmaceutical), GIT medium (manufactured by Nippon Seiyaku), EX-CELL302 medium (manufactured by JRH Biosciences), IMDM medium (manufactured by Invitrogen), hybridoma-SFM medium (manufactured by Invitrogen), these mediums containing additives such as fetal bovine serum (FBS), and the like can be used. The humanized antibody can be expressed and accumulated in the culture supernatant by culturing the resulting transformant in the medium. The expression amount of the humanized antibody in the culture supernatant and the antigen-binding activity thereof can be measured by ELISA described in 1.(4) or the like. The transformant can increase the amount of the humanized antibody produced using a DHFR gene amplification system or the like (Shitara K. et al., J. Immunol. Methods, 167, 271-278, 1994).

The humanized antibody can be purified from the culture supernatant of the transformant using protein A column (Antibodies: A Laboratory Manual, chapter 8; Goding J. W., Monoclonal Antibodies: Principles and Practice, Academic Press, 1996). Further, an ordinary purification method used in proteins is also available. For example, it can be purified by a combination of gel filtration, ion exchange chromatography, ultrafiltration and the like. The molecular weights of H chain, L chain and the whole antibody molecular weight of the purified humanized antibody are measured by the polyacrylamide gel electrophoresis (SDS-PAGE; laemmli U. K. Nature, 227, 680-685, 1970), the Western blotting method (Antibodies: A Laboratory Manual, chapter 12; Goding J. W., Monoclonal Antibodies: Principles and Practice, Academic Press, 1996) or the like.

The antigen-binding activity of the purified humanized antibody can be measured by the enzyme immunoassay using the purified humanized antibody as the first antibody and the labeled anti-human immunoglobulin antibody as the second antibody as described in above 1. (2), the surface plasmon resonance (Karlsson R. et al., J. Immunol. Methods, 145, 229-240, 1991) or the like. Whether the neutralizing activity by which to inhibit the FGF-8 activity is retained or not can be confirmed by the cell growth inhibition assay described in 1. (4).

3. Preparation of an antibody fragment

The antibody fragment can be produced by the genetic engineering method or the protein chemical method using the anti-FGF-8 neutralizing monoclonal antibody and the anti-FGF-8 neutralizing humanized antibody described in 1. and 2. Examples of the antibody fragment include Fab, $F(ab')_2$, Fab', scFv, diabody, dsFv and CDR-containing peptide.

(1) Preparation of Fab

Fab can be prepared by treating the anti-FGF-8 neutralizing antibody with a protease, papain. After the treatment with papain, the fragment is passed through protein A column when the original antibody is an antibody of IgG subclass

having the binding activity to protein A, whereby the fragment can be recovered as uniform Fab by being separated from IgG molecules or Fc fragments (Goding J. W., Monoclonal Antibodies: Principles and Practice, Academic Press, 1996). In case of an antibody of IgG subclass having no binding activity for protein A, Fab can be recovered from a fraction eluted at a low salt concentration by ion exchange chromatography (Goding J. W., Monoclonal Antibodies: Principles and Practice, Academic Press 1996). Further, Fab can be prepared by the genetic engineering method using Escherichia coli. For example, a Fab expression vector can be constructed by cloning the DNAs encoding V regions of the antibodies described in 2. (2), (5) and (6) in a vector for expression of Fab. As the vector for expression of Fab, any of vectors capable of inserting and expressing DNAs for Fab can be used. Examples thereof include pIT106 (Better M. et al., Science, 240, 1041-1043, 1988) and the like. It is possible that the Fab expression vector is introduced into appropriate Escherichia coli and Fab is produced and accumulated in an inclusion body or a periplasmic space. Active Fab can be obtained from the inclusion body by the refolding method which is commonly used in proteins. When Fab is expressed in periplasmic space, active Fab is leaked in the culture supernatant. After the refolding or from the culture supernatant, uniform Fab can be purified using a column immobilized with an antigen (Borrebeck K., Antibody Engineering: A practical Guide, Oxford University Press, 1991).

(2) Preparation of $F(ab')_2$

$F(ab')_2$ can be prepared by treating the anti-FGF-8 neutralizing antibody with a protease, pepsin. After the treatment with pepsin, the fragment can be recovered as uniform $F(ab')_2$ by the same purification procedure as used in Fab (Goding J. W., Monoclonal Antibodies: Principles and Practice, Academic Press, 1996). Further, it can also be prepared by a method in which Fab' described in 3. (3) is treated with a maleimide such as N,N'-o-phenylenedimaleimide or bismaleimidehexane to form thioether linkage or a method in

which it is treated with 5,5'-dithiobis(2-nitrobenzoic acid; DTNB) to form disulfide bond (McCafferty J. et al., Antibody Engineering: A Practical Approach, IRL Press, 1996).

(3) Preparation of Fab'

Fab' can be prepared by treating $F(ab')_2$ described in 3.(2) with a reducing agent such as dithiothreitol. Further, Fab' can also be prepared by the genetic engineering method using Escherichia coli. For example, a Fab' expression vector can be prepared by cloning the DNAs encoding V regions of the antibodies described in 2. (2), (5) and (6) in a vector for expression of Fab'. As the vector for expression of Fab', any of vectors capable of inserting and expressing the DNAs encoding V regions of the antibodies described in 2. (2), (5) and (6) can be used. Examples thereof include pAK19 (Carter P. et al., Bio/technology, 10, 163-167, 1992) and the like. It is possible that the Fab' expression vector is introduced into appropriate Escherichia coli and Fab' is produced and accumulated in an inclusion body or a periplasmic space. Active Fab' can be obtained from the inclusion body by the refolding method which is commonly used in proteins. When Fab' is expressed in the periplasmic space, cells can be disrupted by treatment such as partial digestion with lysozyme, osmotic shock or sonication and recovered extracellularly. After the refolding or from the disrupted cell solution, uniform Fab' can be purified using protein G column or the like (McCafferty J. et al., Antibody Engineering: A Practical Approach, IRL Press, 1996).

(4) Preparation of scFv

scFv can be prepared by the genetic engineering method using phages or Escherichia coli. For example, the DNAs encoding VH and VL of the antibodies described in 2. (2), (5) and (6) are linked through a DNA encoding a polypeptide linker comprising an amino acid sequence of 12 residues or more to produce a DNA encoding scFv. It is important that the polypeptide linker is optimized such that its addition does not inhibit the binding of VH and VL to an antigen. For example, a linker indicated by Pantoliano et al. (Pantoliano M. W. et

al., Biochemistry, 30, 10117-10125, 1991) or a variant thereof can be used.

An scFv expression vector can be prepared by cloning the produced DNA into a vector for expression of scFv. As the vector for expression of scFv, any of vectors capable of incorporating and expressing the DNA of scFv can be used. Examples thereof include pCANTAB5E (manufactured by Amersham Biosciences), Phfa (Lah M. et al., Hum. Antibodies Hybridomas, 5, 48-56, 1994) and the like. The scFv expression vector is introduced into appropriate Escherichia coli, and infected with a helper phage, whereby a phage can be obtained in which scFv is expressed on the surface of the phage by being fused with the phage surface protein. Further, scFv can be produced and accumulated in an inclusion body or a periplasmic space of Escherichia coli having introduced therein the scFv expression vector. From the inclusion body, active form of scFv can be formed by the refolding method which is commonly used in proteins. When it is expressed in the periplasmic space, cells can be disrupted by treatment such as partial digestion with lysozyme, osmotic shock or sonication and recovered extracellularly. After the refolding or from the disrupted cell solution, uniform scFv can be purified using cation exchange chromatography or the like (McCafferty J. et al., Antibody Engineering: A Practical Approach, IRL Press, 1996).

(5) Preparation of diabody

The diabody can be prepared such that the polypeptide linker in the preparation of scFv is produced of 3 to 10 residues. In case of using VH and VL of one type of the antibody, a divalent diabody can be produced. In case of using VHs and VLs of two types of the antibodies, a diabody having di-specificity can be produced (Le Gall F. et al., FEBS Lett., 453, 164-168, 1999, Courage C. et al., Int. J. Cancer, 77, 763-768, 1998).

(6) Preparation of dsFv

dsFv can be prepared by the genetic engineering method using Escherichia coli. First, mutation is introduced into appropriate sites of the DNAs encoding VH and VL of the antibodies

described in 2. (2), (5) and (6) to produce DNAs in which an amino acid residue encoded is replaced with cysteine. The modification of the amino acid residue with the cysteine residue can be performed by the method for mutagenesis using PCR as described in 2. (6). The respective DNAs produced are cloned into the vector for expression of dsFv to produce the expression vector of VH and VL. As the vector for expression of dsFv, any of vectors capable of inserting and expressing DNAs for dsFv can be used. Examples thereof include pUL19 (Reiter Y. et al., Protein Eng., 7, 697-704, 1994) and the like. The expression vector of VH and VL is introduced into appropriate Escherichia coli, and VH and VL can be formed and accumulated in an inclusion body or a periplasmic space. From the inclusion body or the periplasmic space, VH and VL are obtained, and mixed. Disulfide bonds are provided by the refolding method which is commonly used in proteins to form active dsFv. After the refolding, the fragment can further be purified by ion exchange chromatography, gel filtration or the like (Reiter Y. et al., Protein Eng., 7, 697-704, 1994).

(7) Preparation of a CDR-containing peptide

The CDR-containing peptide can be prepared by a chemical synthesis method such as the Fmoc method, the tBoc method or the like. A CDR-containing peptide expression vector can be prepared by producing a DNA encoding the CDR-containing peptide and cloning the resulting DNA into an appropriate vector for expression. As the vector for expression, any of vectors capable of inserting and expressing the DNA encoding the CDR-containing peptide can be used. Examples thereof include pLEX (manufactured by Invitrogen), pAX4a+ (manufactured by MoBiTec) and the like. The expression vector is introduced into appropriate Escherichia coli, and the CDR-containing peptide can be produced and accumulated in an inclusion body or a periplasmic space. From the inclusion body or the periplasmic space, the CDR-containing peptide can be obtained, and purified by ion exchange chromatography, gel filtration or the like (Reiter Y. et al., Protein Eng., 7, 697-704, 1994).

(8) Evaluation of activity

The antigen-binding activity of the antibody fragment can be measured by the enzyme immunoassay using the antibody fragment as the first antibody as described in 1. (2), the surface plasmon resonance (Karlsson R. et al., J. Immunol. Methods, 145, 229-240, 1991) or the like. Further, whether the neutralizing activity by which to inhibit the FGF-8 activity is retained or not can be confirmed by the cell growth inhibition assay described in 1. (4).

4. Preventing or treating agent of the present invention

The anti-FGF-8 neutralizing antibody becomes a cartilage protecting agent because the antibody has such an ability that it is bound to FGF-8 in cells and tissues of a synovial membrane or a cartilage to inhibit the degradation of extracellular matrix of the cartilage induced by FGF-8 and the destruction of the cartilage. Since the antibody has an ability to inhibit the growth of synovial cells induced by FGF-8, it becomes an agent for inhibiting growth of synovial cells. Since the destruction of joints involves the destruction of the cartilage and the growth of synovial cells, the antibody becomes an agent for inhibiting joint destruction by inhibiting the growth of synovial cells and the destruction of the cartilage. Since the arthritis is a disease with the joint destruction, the antibody becomes an agent for treating and preventing arthritis by inhibiting the joint destruction. Examples of the arthritis include osteoarthritis, rheumatoid arthritis, systemic lupus erythematosus, ankylotic arthropathy, psoriatic arthritis, intervertebral disc disease, acute crystalline synovitis (gout, pseudogout) and the like.

Since the humanized antibody comprises a major part derived from the amino acid sequence of the human antibody in comparison to the monoclonal antibody of the non-human animal, it is expected that the high effect is shown within the human body, the immunogenicity is low and its effect is maintained over a long period of time. Thus, the humanized antibody is

preferable as the preventing or treating agent.

The agent comprising the anti-FGF-8 neutralizing antibody may be administered as the treating agent alone. However, it is usually preferable to provide the agent as a pharmaceutical formulation produced by mixing the agent with one or more pharmaceutically acceptable carriers according to any method well known in the technical field of pharmaceutical.

As the administration route, it is advisable to use the most effective route in the treatment. Examples thereof can include oral administration and parenteral administrations such as intraoral, intratracheal, intrarectal, subcutaneous, intramuscular, intraarticular and intravenous administrations. In case of the antibody or peptide preparations, intraarticular and intravenous administrations are preferable.

Examples of the administration form include sprays, capsules, tablets, granules, syrups, emulsions, suppositories, injections, ointments, tapes and the like.

Examples of appropriate preparations for oral administration include emulsions, syrups, capsules, tablets, powders, granules and the like.

Liquid preparations such as emulsions and syrups can be produced by using, as additives, water, saccharides such as sucrose, sorbitol and fructose, glycols such as polyethylene glycol and propylene glycol, oils such as sesame oil, olive oil and soybean oil, antiseptics such as p-hydroxybenzoic acid esters, and flavors such as strawberry flavor and peppermint.

Capsules, tablets, powders, granules and the like can be produced by using, as additives, excipients such as lactose, glucose, sucrose and mannitol, disintegrating agents such as starch and sodium alginate, lubricants such as magnesium stearate and talc, binders such as polyvinyl alcohol, hydroxypropyl cellulose and gelatin, surfactants such as fatty acid esters, and plasticizers such as glycerin.

Examples of preparations appropriate for parenteral administration include injections, suppositories, sprays and the like.

Injections are prepared by using a carrier comprising a salt solution, a glucose solution or a mixture of both, and the like.

Suppositories are prepared using a carrier such as cacao butter, hydrogenated fat or carboxylic acid.

Sprays are prepared by using the antibody or the peptide as such or in combination with a carrier which facilitates dispersion and absorption of the antibody or the peptide in the form of fine particles without stimulating the mouth and the airway mucous membrane of a recipient.

Specific examples of the carrier include lactose, glycerin and the like. Preparations such as aerosol and dry powder can be formed depending on properties of the antibody or the peptide and the carrier used. These parenteral preparations may comprise the ingredients listed as additives in the oral preparations.

The dose or the number of administrations varies with the desired therapeutic effects, the administration method, the therapeutic period, the age, the body weight and the like. It is usually from 10 μg/kg to 20 mg/kg per day for an adult.

Whether the anti-FGF-8 neutralizing antibody inhibits the degradation of extracellular matrix of the cartilage and the growth of synovial cells can be confirmed using the in vitro assay system described in (1) and (2) below. Further, whether the anti-FGF-8 neutralizing antibody becomes the agent for treating or preventing arthritis can be evaluated by administering the antibody to arthritis morbid state model animals described in (3) below and examining whether it can reduce the arthritic syndromes thereof.

(1) Inhibitory activity to cartilage destruction by FGF-8

The cartilage destruction can be evaluated by the assay indicating the degradation of extracellular matrix of the cartilage using chondrocytes or cartilaginous organs and the increase in production of destruction factors from chondrocytes and synovial cells, and the destruction of the subchondral bone according to the progression of the cartilage destruction can

be evaluated by the assay indicating the bone resorption amount respectively.

(a) Degradation of extracellular matrix of the cartilage

The function of the cartilage destruction can be evaluated by culturing rabbit articular chondrocytes subjected to primary culture in the presence of FGF-8 in case of adding the anti-FGF-8 neutralizing antibody and in case of not adding the same and measuring the amount of extracellular matrix remaining on the plate after the culturing. The amount of extracellular matrix is measured in terms of the amount of glycosaminoglycan liberated by the papain treatment. When the decrease in extracellular matrix induced by FGF-8 is inhibited by the addition of the anti-FGF-8 neutralizing antibody, the antibody is considered to have the inhibitory activity of cartilage destruction.

The function of the cartilage destruction can also be evaluated by culturing the cartilaginous organ of the bovine nasal septum subjected to primary culture according to the method of Price et al., (Price J. S. et al., Arthritis Rheum., 42, 137-147, 1999) in the presence of FGF-8 in case of adding the anti-FGF-8 neutralizing antibody and in case of not adding the same and measuring the amount of extracellular matrix in the cartilaginous organ after the culturing. When the decrease in extracellular matrix induced by FGF-8 is inhibited by the addition of the anti-FGF-8 neutralizing antibody, the antibody is considered to have the inhibitory activity of cartilage destruction. The amount of extracellular matrix is measured by treating the organ after the culturing with papain and measuring the amount of released glycosaminoglycan by the dimethylene blue method (Chandrasekhar S. et al., Anal. Biochem. 161 103-108, 1987) or measuring the amount of collagen in terms of a concentration of hydroxyproline according to Tokyo Eisei Nenpo, 36, 277, 1985.

(b) Production of factors involved in cartilage destruction

Examples of the factor involved in cartilage destruction can include prostaglandin E_2, matrix metalloproteinase-3 and nitric oxide. Rabbit joint chondrocytes or rabbit synovial

cells are cultured in the presence of FGF-8 in case of adding the anti-FGF-8 neutralizing antibody and in case of not adding the same, and prostaglandin E_2, matrix metalloproteinase-3 or nitric oxide in the culture supernatant is measured as the amount of these factors produced from the cells. When the production of prostaglandin E_2, matrix metalloproteinase-3 or nitric oxide promoted by FGF-8 is inhibited by the addition of the anti-FGF-8 neutralizing antibody, the antibody is considered to have the inhibitory activity of cartilage destruction.

Prostaglandin E_2 can be measured by Prostaglandin E_2 EIA system (manufactured by Amersham Biosciences), matrix metalloproteinase-3 can be measured by Rabbit Matrix Metalloproteinase-3 ELISA system (manufactured by Amersham Biosciences) and nitric oxide can be measured by the method using Griess reagent (Green L. C. et al., Anal. Biochem. 126, 131-138 1982).

(c) Bone resorption

The bone resorption can be evaluated by culturing the mouse calvariae in the presence of FGF-8 in case of adding the anti-FGF-8 neutralizing antibody and in case of not adding the same according to the method of Kusano et al. (Kusano K., et al., Endocrinology, 139, 1338-1345, 1998) and measuring the concentration of calcium or the concentration of hydroxyproline in the culture supernatant. When the bone resorption promoted with FGF-8 is inhibited by the addition of the anti-FGF-8 neutralizing antibody, the antibody is considered to have the inhibitory activity of cartilage destruction. The concentration of calcium in the culture supernatant can be measured by Calcium C-Test Wako (manufactured by Wako Pure Chemical Industries, Ltd.). The concentration of hydroxyproline in the culture supernatant can be measured according to Tokyo Eisei Nenpo, 36, 277, 1985.

(2) Synovial cell growth inhibitory activity

The growth of synovial cells can be evaluated by culturing synovial cells of human or rabbit in the presence of FGF-8 in case of adding the anti-FGF-8 neutralizing antibody and in case

of not adding the same and measuring the amount of incorporation of [^{3}H]thymidine. When the amount of incorporation of [^{3}H]thymidine promoted by FGF-8 is inhibited by the addition of the anti-FGF-8 neutralizing antibody, the antibody is considered to have the synovial cell growth inhibitory activity.

(3) in vivo evaluation using arthritis model in animals

The effect of FGF-8 or the anti-FGF-8 neutralizing antibody on the joint destruction can be evaluated using the following arthritis model. The anti-FGF-8 neutralizing antibody is administered to the arthritis model. When the arthritis in the model animals is ameliorated, the antibody is considered to be available as the agent for treating or preventing arthritis.

Examples of the model animals showing the symptoms similar to rheumatoid arthritis can include MRL-lpr/lpr mouse (Hang L. et al., J. Exp. Med., 155, 1690-1701, 1982, the mouse can be purchased from Japan Charles River) in which arthritis is spontaneously triggered mainly on the leg joint, rat adjuvant arthritis model (Taurog J. D. et al., Cell. Immunol., 75, 271-282, 1983) with arthritis induced by immunization with dead tubercule bacillus, mouse collagen-induced arthritis model (Stuart J. M. et al., Annu. Rev. Immunol., 2, 199-218, 1984) with arthritis induced by immunization with type II collagen often found in joints along with an adjuvant, and the like. These model animals show the symptoms similar to rheumatoid arthritis, and are widely used in evaluation of the therapeutic drugs of arthritis.

When the rat adjuvant arthritis model is used, the volumes of the hind paw edema are measured with as time goes by. Both the hind paws are subjected to soft X-ray photography to evaluate the bone destruction and the deformation of joints. With respect to the index of the systemic responce of inflammation, the concentration of mucoprotein in serum is measured using Aspro-GP (manufactured by Otsuka Seiyaku), and the concentration of nitric oxide in serum by the method of Tracey et al. (Tracey W. R., et al., J. Pharmacol. Exp. Ther., 272, 1011-1015, 1995) respectively.

In case of using the mouse collagen-induced arthritis model, the change in body weight and the arthritic score of all limbs as time goes by and the anti-collagen antibody titer in serum are measured. Further, after dissection, the histopathological examination of the joint is performed. The arthritis is evaluated by scoring of 0 to 4 in one limb and 16 at the highest in all limbs. The scoring criteria are; 0: normal, 1: weak erythema is observed, 2: weak swelling and erythema are observed, 3: strong swelling and erythema are observed and warmth is felt by touch, and 4: clear swelling with deformation of fingers is observed.

As the osteoarthritis model, a model of a large animal such as a dog or a rabbit of which the joint is loosened by excising the meniscus of the knee or separating the ligament to cause chronic degeneration of the joint (hereinafter referred to as an experimental osteoarthritis model) has been often used (Ito Ryuta, Shinyaku Kaihatsu no tameno Dobutsu Moderu Riyo Shusei, Henkeisei Kansetsusho, R & D Planning, 1985, Guingamp C. et al., Arthritis Rheum., 40, 1670-1679, 1997, van der Kraan P. M. et al., Am. J. Pathol., 135, 1001-1014, 1989). Further, a monoiodoacetic acid-induced osteoarthritis rat model in which monoiodoacetic acid is injected is also listed as the osteoarthritis model.

The experimental osteoarthritis model obtained by partial excision of the rabbit knee joint meniscus can be produced by the method of Colombo et al. (Colombo C. et al., Arthritis Rheum., 26, 875-886, 1983) and the method of Kikuchi et al (Kikuchi Sumiyuki et al., Kansetsu Geka, 15, 92-98, 1966).

The monoiodoacetic acid-induced osteoarthritis rat model can be produced by injecting monoiodoacetic acid into the rat knee joint according to the method of Guingamp et al. (Guingamp C. et al., Arthritis Rheum., 40, 1670-1679, 1997).

In the osteoarthritis model animals, the knee joint patella is extracted after a certain period of time, and treated with papain, and the amount of glycosaminoglycan is measured by the dimethylmethylene blue method (Chandrasekhar S. et al.,

Anal. Biochem. 161, 103-108, 1987) to evaluate the joint destruction (degradation of extracellular matrix). Further, the histopathological examination of the knee joint is performed.

The dosage form and the administration route in administering the anti-FGF-8 neutralizing antibodies to the model animals can properly be selected depending on the qualities of the objective model animals and the severity. For example, these can be administered to the models animal orally or parenterally (intraperitoneal, intravenous, intraarticular, intramuscular or subcutaneous administration) either as such or in combination with other pharmatheutically acceptable additives such as carriers, excipients and diluents.

The mixing amount and the dose of the anti-FGF-8 neutralizing antibody are individually determined depending on the administration method, the dosage form and the use purpose of the preparations, the specific symptoms of the model animal, the body weight of the model animal and the like, and these are not particularly limited. The administration is possible with a dose of approximately 1 μg/kg to 100 mg/kg per day and once a day as an administration interval. The administration is also possible from two to four times a day, or more times a day. Further, the continuous administration through drip infusion or the like is also possible. When the antibody is administered to parts such as joints, it is administered to one position at a dose of from approximately 1 pg to 100 mg.

5. Diagnostic agent of the present invention

FGF-8 induces the growth of synovial cells in joints and the destruction of extracellular matrix in cartilages. The foregoing anti-FGF-8 antibody can specifically bind to FGF-8 to detect and determine FGF-8. Thus, it can be used as a diagnostic agent of arthritis. Examples of the arthritis that can be diagnosed include the diseases described in 4. above. The detection and the determination of FGF-8 can be performed by the method described in 6. below.

As the anti-FGF-8 antibody used in the diagnostic agent of the present invention, any of antibodies which specifically bind to FGF-8 can be used. A monoclonal antibody and a polyclonal antibody are both available. A monoclonal antibody is preferably used.

Examples of the monoclonal antibody include an antibody produced by a hybridoma, a humanized antibody and an antibody fragment of these antibodies.

The anti-FGF-8 antibody used in the diagnostic agent of the present invention can be produced similarly by the method of producing the anti-FGF-8 neutralizing antibody. However, it is not required to inhibit activity of FGF-8. The anti-FGF-8 neutralizing antibody can also be used as the anti-FGF-8 antibody used in the diagnostic agent of the present invention. Specific examples of the anti-FGF-8 antibody used in the diagnostic agent of the present invention include monoclonal antibody KM1334 produced by hybridoma KM1334 (FERM BP-5451) and human chimeric antibody KM3034 produced by transformant KM3034 (FERM BP-7836).

The diagnostic agent comprising the anti-FGF-8 antibody may comprise a reagent for conducting an antigen-antibody reaction according to a diagnosing method indicated in 6. below and a detection reagent of the reaction. Examples of the reagent for performing the antigen-antibody reaction include buffer solutions, salts and the like. Examples of the detection reagent include reagents used in a usual immunological detection method, such as a labeled second antibody that recognizes the anti-FGF-8 antibody and a substrate corresponding to a label.

6. Method for diagnosing arthritis in the present invention

Examples of the arthritis which is diagnosed by the diagnosing method of the present invention include the diseases listed in 4. above. It is considered that in the joints of patients suffering from these diseases, the amount of FGF-8 having the activity of inducing the growth in synovial cells and the destruction of extracellular matrix in the cartilage is increased in comparison to healthy persons.

The method for diagnosing arthritis of the preset

invention includes, for example, a method in which FGF-8 present in cells or tissues is immunologically detected and/or determined as described below using cells or tissue sections of the synovial membrane or the cartilage in the joint collected from subjects by the biopsy or the like and the cell extract or the synovial fluid produced from the cells or the tissues.

As the method for immunologically detecting and/or determining FGF-8 expressed in the joint using the anti-FGF-8 antibody, the fluorescent antibody method, the enzyme immunoassay (ELISA), the radio immunoassay (RIA), the immunotissue staining method, the immunocyte staining method, the Western blotting method, the immuno-precipitation method, the sandwich ELISA method (Tomiyama Sakuji & Ando Tamie, Tankuron Kotai Jikken Manual, Kodansha Scientific, 1987, Nihon Seikagaku Kai, Zoku Seikagaku Jikken Koza 5, Men-eki Seikagaku Kenkyuho, Tokyo Kagaku Dojin, 1986) and the like can be used.

The fluorescent antibody method can be performed by the method described in a document (Monoclonal Antibodies, Tomiyama Sakuji & Ando Tamie, Tankuron Kotai Jikken Manual, Kodansha Scientific, 1987) or the like. Specifically, a cell or a tissue of a joint isolated is reacted with the anti-FGF-8 antibody and further with an anti-immunoglobulin antibody labeled with a fluorescent substance such as fluorescein isothiocyanate (FITC) or phycoerythrin and the fluorescent dye is then measured with a flow cytometer.

The enzyme immunoassay (ELISA) is a method in which isolated cells, tissues, synovial fluids or the like from a joint are reacted with the anti-FGF-8 antibody and further reacted with an anti-immunoglobulin antibody labeled with an enzyme such as peroxidase or alkaline phosphatase, a substrate formed by an enzyme reaction is added for reaction, and the developed dye is measured by a spectrophotometer.

The radio immunoassay (RIA) is a method in which isolated cells, tissues, synovial fluids or the like from a joint are reacted with the anti-FGF-8 antibody and further reacted with an anti-immunoglobulin antibody labeled with an radioisotope,

and the radioactivity is then measured with a scintillation counter or the like.

The immunocyte staining method and the immunotissue staining method are methods in which isolated cells, tissues, disrupted solutions thereof, synovial fluids or the like from a joint are reacted with the anti-FGF-8 antibody and further reacted with an anti-immunoglobulin antibody labeled with a fluorescent substance such as FITC, an enzyme such as peroxidase or alkaline phosphatase, or the like and a substrate developed by an enzyme reaction is added for reaction in case of labeling with an enzyme, after which observation with a microscope is performed. It can be performed by the method described in a document (Goding J.W., Goding J. W., Monoclonal Antibodies: Principles and Practice, Academic Press, 1996; Toyama Sakuji & Ando Tamie, Tan Kuron Kotai Jikken Manual, Kodansha Scientific, 1987).

The Western blotting is a method in which isolated cells, tissues, disrupted solutions thereof, synovial fluids or the like from a joint are dissolved in a sample buffer solution containing SDS to perform SDS-polyacrylamide gel electrophoresis, the resulting sample is then transferred on a polyvinylidene fluoride (PVDF) film, and reacted with the anti-FGF-8 antibody and further reacted with an anti-immunoglobulin antibody labeled with an enzyme such as peroxidase or alkaline phosphatase, after which the reaction product is reacted with a substrate developed by an enzyme reaction or a substrate chemically illuminated and is detected as bands.

The immuno-precipitation method is a method in which disrupted cells, tissue solutions or a synovial fluid isolated from a joint are reactrd with the anti-FGF-8 antibody immobilized on beads or the like are reacted, the beads are isolated by centrifugation or the like and then treated with an SDS-comprising sample buffer, and dissolved FGF-8 is detected by the Western blotting or the like.

Sandwich ELISA is one of the enzyme immunoassays using

two types of anti-FGF-8 antibodies different in epitope. It is a method in which one of the anti-FGF-8 antibodies is immobilized on a plate, and reacted with isolated cells, tissues, disrupted solutions thereof or synovial fluids from a joint, after which FGF-8 bound to the anti-FGF-8 antibody on the plate is further reacted with the other anti-FGF-8 antibody. The sample is reacted with an anti-immunoglobulin antibody labeled with an enzyme such as peroxidase or alkaline phosphatase and further with a substrate developed by an enzyme reaction, and the formed dye is measured with a spectrophotometer.

Examples and Reference Examples of the present invention are described below.

Example 1

Degradation of extracellular matrix of rabbit chondrocytes with FGF-8 and inhibition thereof by an antibody

Rabbit articular chondrocytes were isolated from both knees and shoulders of 3-week-old New Zealand white female rabbits and cultured according to the method of Tamura et al. (Tamura T. et al., Eur. J. Pharmacol., 419, 269-274, 2001). That is, the joints of both knees and the joints of both shoulders were isolated to collect the epiphyseal cartilages. These cartilages were washed with a phosphate-buffered saline solution, then sliced, and treated with 10 vol% FBS-comprising DMEM (FBS-comprising DMEM is hereinafter referred to as FBS/DMEM) comprising 0.4 w/v% actinase E at 37°C for 1 hour and further with 10 vol% FBS/DMEM comprising 0.025 w/v% collagenase P at 37°C for 5 to 6 hours to isolate and collect chondrocytes from the cartilaginous tissues. The collected chondrocytes were suspended in 10 vol% FBS/DMEM and adjusted to 100,000 cells/mL. The culture solution comprising the chondrocytes was inoculated in each well of a 24-well plate in an amount of 1 mL, and cultured in a gaseous phase of 5% CO_2-95% air at 37°C. After the chondrocytes became confluent, the culture medium was replaced with 0.5 vol% FBS/DMEM, followed by the culturing for 24 hours. The culture medium was removed,

and 1 mL of 0.5 vol% FBS/DMEM (control; non-stimulated), 0.5 vol% FBS/DMEM comprising FGF-8 (1, 10 or 100 ng/mL; manufactured by Peprotech), 0.5 vol% FBS/DMEM comprising FGF-8 (100 ng/mL), or 1 mL of 0.5 vol% FBS/DMEM comprising FGF-8 (100 ng/mL) and anti-FGF-8 antibody KM1334 (1, 3 or 10 μg/mL) were added, followed by the culturing for 48 hours. The culture solution was removed, and the amount of glycosaminoglycan in extracellular matrix remaining on the plate was measured by the dimethymethylene blue (DMMB) method (Chandrasekhar S. et al., Anal. Biochem. 161 103-108, 1987). That is, papain (manufactured by Sigma-Aldrich) was added to a storagebuffer of papain (0.1 mol/L sodium acetate, 50 mmol/L EDTA, pH 5.8) activated by adding 5 mmol/L-cysteine hydrochloride monohydrate to a final concentration of 20 μg/ml. Each well of the plate on which the chondrocytes had been cultured was added 1 mL of this solution, and digested overnight at 60°C. To 75 μL of this digested solution were added 25 μL of a guanidine hydrochloride buffer (2.88 mol/L guanidine hydrochloride, 50 mmol/L sodium acetate, pH 6.8) and 200 μL of a DMMB solution, and the absorbance was measured at 530/590 nm. The concentration of glycosaminoglycan of each sample was calculated from the absorbance of chondroitin sulfate (derived from shark cartilages, manufactured by Seikagaku Kogyo) used as a standard. Three experiments were performed for each condition and the mean values and the standard error (SE) were calculated.

The results are shown in Figs 1 and 2. FGF-8 significantly decreased the residual amount of glycosaminoglycan in extracellular matrix at the concentration of 100 ng/mL (Fig. 1). This shows that FGF-8 has activity of promoting degradation of extracellular matrix of the cartilage. Further, anti-FGF-8 neutralizing antibody KM1334 significantly inhibited the promotion of degradation of extracellular matrix of the cartilage with FGF-8 at the antibody concentration of 3 μg/mL or more (Fig. 2). Accordingly, the degradation of extracellular matrix in arthritis can be suppressed by the

administration of the anti-FGF-8 neutralizing antibody.

Example 2

Promotion of growth of rabbit synovial cells with FGF-8 and inhibition thereof by an antibody

Rabbit synovial cells were collected by the method of Hamilton et al. (Hamilton J. A. and Slywka J., J. Immunol. 126, 851-855, 1981). The isolated synovial cells were suspended in RPMI 1640 medium comprising 10 vol% FBS (FBS-comprising RPMI 1640 medium is hereinafter referred to as FBS/RPMI 1640), and 10,000 cells was inoculated at each well of a 96-well culture plate. After 24 hours of the culturing, the culture medium of each well was removed, and 0.2 vol% FBS/RPMI 1640 (control; non-stimulated), and 0.2 vol% FBS/RPMI 1640 comprising FGF-8 (100 ng/mL) and anti-FGF-8 antibody KM1334 (1, 3 or 10 μg/mL) were added to each well. After 48 hours of the culturing, 9.25 kBq per well of [^{3}H]thymidine was added. The culturing was further performed for 24 hours, and the radioactivity of [^{3}H]thymidine incorporated in the cells was measured. Six experiments were performed for each condition, and the mean values and the standard error (SE) were calculated.

The results are shown in Figs. 3 and 4. FGF-8 significantly promoted the incorporation of [^{3}H]thymidine into the rabbit synovial cells at the concentration of 100 ng/mL (Fig.3). This shows that FGF-8 has activity of promoting the growth of the rabbit synovial cells. Further, anti-FGF-8 neutralizing antibody KM1334 significantly inhibited the promotion of the FGF-8-dependent incorporation of [^{3}H]thymidine from the antibody concentration at 0.3 μg/mL (Fig. 4). Accordingly, the administration of the anti-FGF-8 neutralizing antibody can inhibit the growth of the synovial membrane in arthritis.

Example 3

Promotion of growth of human synovial cells with FGF-8 and inhibition thereof by an antibody

The same experiment as in Example 2 was performed using human synovial cells derived from the rheumatoid patient (procured from Toyobo). The concentration of FGF-8 was used at 10, 100 or 500 ng/mL, and the concentration of FGF-8 coexisting along with anti-FGF-8 neutralizing antibody KM1334 was used at 500 ng/mL.

The results are shown in Figs. 5 and 6. FGF-8 significantly promoted the incorporation of [^{3}H]thymidine into the human synovial cells at the concentration of 500 ng/mL (Fig. 5). This shows that FGF-8 has activity of promoting the growth of the human synovial cells. Further, anti-FGF-8 neutralizing antibody KM1334 significantly inhibited the promotion of the FGF-8-dependent incorporation of [^{3}H]thymidine from the antibody concentration at 1 μg/mL (Fig. 6). Accordingly, the administration of the anti-FGF-8 neutralizing antibody can inhibit the growth of the synovial membrane in arthritis.

Example 4

Staining of a synovial membrane using an anti-FGF-8 antibody

Paraffin sections were prepared from a synovial membrane extracted from the human rheumatoid arthritis patient by the method of a document (Tanaka A. et al., Cancer Res. 58, 2053-2056, 1998), and the tissue immunostaining was performed using anti-FGF-8 antibody KM1334. As a result, the synovial cells of the three of four human rheumatoid arthritis synovial membranes were positive for FGF-8. Thus, FGF-8 was confirmed to be present in the human synovial membrane. Further, it was indicated that the human rheumatoid arthritis can be diagnosed by detecting the synovial cells of the human rheumatoid arthritis using the anti-FGF-8 antibody.

Reference Example 1

Preparation of an anti-FGF-8 neutralizing chimeric antibody

1. Isolation and analysis of cDNA encoding the V region of a

mouse anti-FGF-8 neutralizing antibody

(1) Preparation of mRNA from a hybridoma cell producing a mouse anti-FGF-8 neutralizing antibody

Using FastTrack mRNA Isolation Kit (manufactured by Invitrogen) as a mRNA preparation kit according to the attached instruction manual, about 8 μg of mRNA was prepared from 1×10^7 cells of a hybridoma KM1334 (FERM BP-5451) producing an anti-FGF-8 neutralizing monoclonal antibody of mouse (hereinafter called a mouse anti-FGF-8 neutralizing antibody).

(2) Preparation of cDNA libraries for the H chain and the L chain of the mouse anti-FGF-8 neutralizing antibody

Using TimeSaver cDNA Synthesis Kit (manufactured by Amersham Biosciences) according to the attached instruction manual, cDNA with EcoRI-NotI adapters at both the termini was synthetically prepared from 5 μg of the mRNA of KM1334, as obtained above in 1(1) of Reference Example 1. After the cDNA thus prepared was dissolved in its entirety in 20 μl of sterile water, the resulting solution was fractionated by agarose gel electrophoresis. About 0.1 μg each of a cDNA fragment of about 1.5 kb corresponding to the H chain of the antibody in the class IgG and a cDNA fragment of about 1.0 kb corresponding to the L chain in the κ class was obtained. Subsequently, 0.1 μg of the cDNA fragment of about 1.5 kb and 0.1 μg of the cDNA fragment of about 1.0 kb were individually conjugated with 1 μg of a vector λZAPII preliminarily digested with a restriction endonuclease EcoRI and subsequently dephosphorylated of the termini with an alkaline phosphatase derived from calf small intestine, using λZAPII Cloning Kit (manufactured by Stratagene) according to the attached instruction manual. 4 μl each of the individual reaction solutions resulting from the conjugation was packaged into λ phage, using Gigapack II Packaging Extracts Gold (manufactured by Stratagene) according to the attached instruction manual. Then, an appropriate amount of each of the resulting λ phages was infect with an Escherichia coli strain XL1-Blue (manufactured by Stratagene; Biotechniques, 5, 376, 1987), so that about 8.1×10^4 phage

clones and about 5.5×10^4 phage clones were obtained as an H chain cDNA library and an L chain cDNA library, respectively of KM1334. Subsequently, the each phage was immobilized on nylon membranes by the routine method (Molecular Cloning, the third edition).

(3) Cloning of cDNAs for the H chain and L chain of the mouse anti-FGF-8 neutralizing antibody

On the nylon membranes on which the H chain cDNA library and the L chain cDNA library of KM1334 as prepared in 1(2) of Reference Example 1, were immobilized, detection was done using ECL Direct Nucleic Acid Labelling and Detection Systems (manufactured by Amersham Biosciences) according to the attached instruction manual and also using the cDNAs for the C region of the mouse antibody [for the H chain, a DNA fragment containing mouse Cγ1 cDNA (French D. L. et al., J. Immunol., 146, 2010-2016, 1991) was used; and for the L chain, a DNA fragment containing mouse Cκ cDNA (Hieter P. A. et al., Cell, 22, 197-207, 1980) was used] as probes, to individually obtain 10 phage clones strongly binding to the probes for the H chain and the L chain. The each phage clone was then converted into plasmids by in vivo excision according to the manual of the λZAPII Cloning Kit (manufactured by Stratagene). The nucleotide sequence of the cDNA in each of the thus-obtained plasmids was determined using Big Dye Terminator Kit Version 2 (manufactured by Applied Biosystems) according to the dideoxy method (Molecular Cloning, the third edition). As a result, plasmid pKM1334H7-1 comprising the full length functional H chain cDNA and plasmid pKM1334L7-1 comprising the full length functional L chain cDNA in which ATG sequence deduced as initiation codon was present in the 5'-terminal of the cDNA were obtained.

(4) Analysis of the amino acid sequence of the V region of the mouse anti-FGF-8 neutralizing antibody

The full length nucleotide sequence of the VH carried in the plasmid pKM1334H7-1 is represented as SEQ ID No.1, while the speculative whole amino acid sequence thereof is represented as SEQ ID No. 2; the full length nucleotide sequence of the

VL carried in the plasmid pKM1334L7-1 is represented as SEQ ID No. 3, while the speculative full length amino acid sequence is represented as SEQ ID No. 4. In comparison with the sequence data of existing mouse antibodies (Sequences of Proteins of Immunological Interest) and in comparison with the results of the analysis of the N-terminal amino acid sequences of the H chain and the L chain of the purified mouse anti-FGF-8 neutralizing antibody KM1334 by automatic Edman degradation using Protein Sequencer PPSQ-10 (manufactured by Shimadzu Corporation), it was shown that each of the isolated cDNAs was the full-length cDNAs encoding the mouse anti-FGF-8 neutralizing antibody KM1334 with a secretory signal sequence. It was also shown that the sequence of the amino acid residues 1-19 in the amino acid sequence of the H chain as represented as SEQ ID No. 2 was a secretory signal sequence, while the sequence of the amino acid residues 1-19 in the amino acid sequence of the L chain as represented as SEQ ID No. 4 was a secretory signal sequence.

Then, it was examined whether or not the amino acid sequences of the VH and VL of the mouse anti-FGF-8 neutralizing antibody KM1334 (amino acid sequences resulting from the exclusion of their secretory signal sequences) were novel. Screening in the existing protein amino acid sequence database (PIR-Protein Release 56.0) was done by BLAST (Altschul S. F. et al., J. Mol. Biol., 215, 403-410, 1990), using GCG Package (Version 9. 1, manufactured by Genetics Computer Group) as a sequence analysis system. As a result, not any completely identical sequence to the H chain or the L chain was screened for. Thus, it was verified that those of the VH and the VL of the mouse anti-FGF-8 neutralizing antibody KM1334 were novel amino acid sequences.

Furthermore, the CDRs in the VH and the VL of the mouse anti-FGF-8 neutralizing antibody KM1334 were compared with the amino acid sequences of existing antibodies, for identification. The amino acid sequences of the CDR1, CDR2 and CDR3 in the VH of the mouse anti-FGF-8-neutralizing antibody KM1334 are

represented as SEQ ID Nos. 5, 6 and 7, respectively, while the amino acid sequences of the CDR1, CDR2 and CDR3 in the VL of the mouse anti-FGF-8-neutralizing antibody KM1334 are represented as SEQ ID NOS. 8, 9 and 10, respectively.

2. Stable expression of an anti-FGF-8 neutralizing chimeric antibody using an animal cell

(1) Construction of a vector pKANTEX1334 for expression of an anti-FGF-8 neutralizing chimeric antibody

Using the vector pKANTEX93 for expression of humanized antibodies as described in WO 97/10354 and the plasmids pKM1334H7-1 and pKM1334L7-1 as obtained in 1(3) of Reference Example 1, a vector pKANTEX1334 expressing an anti-FGF-8 neutralizing chimeric antibody was constructed as follows.

Using 50 ng of the plasmid pKM1334H7-1 obtained in 1(3) of Reference Example 1, as template, the synthetic DNAs with the nucleotide sequences represented as SEQ ID Nos. 11 and 12 (manufactured by GENSET) were added as primers to a final concentration of 0.3 μmol/L, for PCR in a 50-μl system under conditions such that first heating at 94°C for 2 minutes was followed by 30 cycles of 94°C for 15 second, 55°C for 30 seconds and 68°C for one minute, using KOD plus polymerase (manufactured by TOYOBO) according to the attached instruction manual. After the resulting reaction solution was precipitated in ethanol, the resulting precipitate was dissolved in sterile water, for reaction with 10 units of a restriction endonuclease ApaI (manufactured by Takara Shuzo) and 10 units of a restriction endonuclease NotI (manufactured by New England Biolabs) at 37°C for one hour. The reaction solution was fractionated by agarose gel electrophoresis, to recover about 0.3 μg of an ApaI-NotI fragment of about 0.47 kb.

Subsequently, 3 μg of a vector pKANTEX93 for expression of humanized antibodies reacted with 10 units of a restriction endonuclease ApaI and 10 units of a restriction endonuclease NotI for 37°C for one hour. The reaction solution was fractionated by agarose gel electrophoresis, to recover about

2 μg of an ApaI-NotI fragment of about 12.75 kb.

Then, 0.1 μg of the NotI-ApaI fragment derived from the PCR products and 0.1 μg of the NotI-ApaI fragment derived from the plasmid pKANTEX93 as obtained above were added to sterile water of 10 μl in total, for ligation using Ligation High (manufactured by TOYOBO). The recombinant plasmid DNA solution thus obtained was used to transform an Escherichia coli strain JM109, to obtain a plasmid pKANTEX1334H shown in Fig. 7.

Using 50 ng of the plasmid pKM1334L7-1 obtained in 1(3) of Reference Example 1, as template, the synthetic DNAs with the nucleotide sequences represented as SEQ ID Nos. 13 and 14 (manufactured by GENSET) were added as primers to a final concentration of 0.3 μmol/L, for PCR in a 50-μl system under conditions such that first heating at 94°C for 2 minutes was followed by 30 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 68°C for one minute, using KOD plus polymerase according to the attached instruction manual. After the resulting reaction solution was precipitated in ethanol, the resulting precipitate was dissolved in sterile water, for a reaction with 10 units of a restriction endonuclease EcoRI (manufactured by Takara Shuzo) and 10 units of a restriction endonuclease BsiWI (manufactured by New England Biolabs) at 37°C for one hour. The reaction solution was fractionated by agarose gel electrophoresis, to recover about 0.3 μg of an EcoRI-BsiWI fragment of about 0.44 kb.

Subsequently, 3 μg of the plasmid KANTEX1334H obtained above reacted with 10 units of a restriction endonuclease EcoRI and 10 units of a restriction endonuclease BsiWI for 37°C for one hour. The reaction solution was fractionated by agarose gel electrophoresis, to recover about 2 μg of an EcoRI-BsiWI fragment of about 13.20 kb.

Then, 0.1 μg of the EcoRI-BsiWI fragment derived from the PCR products and 0.1 μg of the EcoRI-BsiWI fragment derived from the plasmid pKANTEX1334H as obtained above were added to sterile water of 10 μl in total, for ligation using Ligation High (manufactured by TOYOBO). The recombinant plasmid DNA

solution thus obtained was used to transform an Escherichia coli strain JM109, to obtain a plasmid pKANTEX1334 shown in Fig. 7.

400 ng of the resulting plasmid was used for the analysis of the nucleotide sequence thereof by the dideoxy method using Big Dye Terminator Kit Version 2 (manufactured by Applied Biosystems). It was confirmed that a plasmid with the intended DNA cloned therein was obtained.

(2) Stable expression of an anti-FGF-8 neutralizing chimeric antibody using an animal cell

Using the vector pKANTEX1334 expressing an anti-FGF-8 neutralizing chimeric antibody as obtained in 2(1) of Reference Example 1, the anti-FGF-8 neutralizing chimeric antibody was expressed in an animal cell as follows.

After 4 μg of the plasmid pKANTEX1334 was introduced into 1×10^6 cells of the DHFR gene-deficient CHO cell (Urlaub G., et al., Cell, 33, 405-412, 1983) by the electroporation method (Miyaji H. et al., Cytotechnology, 3, 133-140, 1990), the resulting cells were suspended in 30 ml of IMDM-1×HT supplement-FBS (10) [IMDM culture medium containing 10 % dialyzed FBS and 1 × HT supplement (both manufactured by Invitrogen)]. Then, the resulting suspension was divided at 100 μl/well in a 96-well microtiter plate (manufactured by Iwaki Glass). After the cells were cultured in a 5% CO2 incubator at 37 °C for 24 hours, the culture broth was replaced with IMDM-FBS (10) (IMDM culture medium containing 10 % dialyzed FBS), for additional one- to two-week culturing. The culture supernatant was recovered from the wells, where resistant colonies developed to became confluent. The antigen-binding activity of the anti-FGF-8 neutralizing chimeric antibody in the supernatant was assayed by ELISA described in 2(3) of Reference Example 1.

The tranformants of the well in which the expression of the anti-FGF-8 neutralizing chimeric antibody was observed in the culture supernatant was inoculated in a 24-well plate. For the purpose of raising the expression of the antibody using

the dhfr gene amplification system, the transformant was cultured in an IMDM-FBS culture medium (10) containing 50 nM of methotrexate (MTX; manufactured by Sigma Aldrich) as an inhibitor of DHFR as a DHFR gene product, for 2 weeks. By raising the MTX concentration to 200 nM and 500 nM, additionally, the transformant was cultured at each of the stages for two weeks, to induce a transformant with resistance against 500 nmol/L MTX. Just when the transformant became confluent in the well, the antigen-binding activity of the anti-FGF-8 neutralizing chimeric antibody in the culture supernatant was assayed by ELISA described in 2(3) of Reference Example 1. Finally, a transformant with an ability of proliferating in the IMDM-FBS (10) containing MTX at a concentration of 500 nM to highly express the anti-FGF-8 neutralizing chimeric antibody was obtained. The resulting transformant was prepared into a single cell (clone preparation), by the limiting dilution method twice, so that a transformant clone with the highest expression of the anti-FGF-8 neutralizing chimeric antibody was designated KM3034. Further, the anti-FGF-8 neutralizing chimeric antibody generated by the transformant clone KM3034 was designated KM3034.

(3) Assaying the activity of the antibody to bind to FGF-8 (ELISA)

FGF-8-b [manufactured by R&D Systems] was dissolved in PBS to 10 μg/ml. The resulting solution was divided in 50-μl portions in each well of a 96-well ELISA plate [manufactured by Greiner]. Then, the plate was left to stand alone at room temperature for two hours, to adsorb FGF-8-b onto the plate surface. After the FGF-8 solution was discarded, PBS containing 1 % BSA (BSA-PBS) was added at 100 μl/well, for one-hour reaction at room temperature so as to block the remaining active groups. Discarding BSA-PBS, various dilutions of the culture supernatant of the transformant, the purified mouse antibody or the purified humanized antibody were added at 50 μl/well, for reaction at room temperature for one hour. After the reaction, the each well was washed with PBS

containing 0.05 % Tween 20 (Tween-PBS). Subsequently, a peroxidase-labeled rabbit anti-mouse Ig antibody solution [manufactured by DAKO] diluted 400-fold with BSA-PBS was added to the wells, to which the mouse antibody was preliminarily added. To the wells to which the humanized antibody was preliminarily added, a peroxidase-labeled goat anti-IgG (H+L) antibody solution diluted 3000-fold with BSA-PBS was added as a secondary antibody solution. These were added at 50 µl/well for reaction at room temperature for one hour. After the reaction, the wells were washed with Tween-PBS, to which an ABTS substrate solution [a solution prepared by dissolving 0.55 g of ammonium 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonate) in 1 L of 0.1 M citrate buffer solution (pH 4.2) and adding hydrogen peroxide at 1 µL/mL immediately before use] was added at 50 µL/well for chromogenic reaction. Then, the absorbance at 415 nm (OD415) was measured, to assay the binding activity of the antibody.

Reference Example 2

Preparation of an anti-FGF-8 neutralizing CDR-grafted antibody

1. Construction of cDNAs encoding the VH and VL of an anti-FGF-8 neutralizing CDR-grafted antibody

(1) Designing the amino acid sequences of the VH and VL of an anti-FGF-8 neutralizing CDR-grafted antibody

First, the amino acid sequence of the VH of an anti-FGF-8 neutralizing CDR-grafted antibody was designed as follows. So as to graft the amino acid sequence of the CDR in the VH of the anti-FGF-8 neutralizing mouse antibody KM1334 identified in 1(4) of Reference Example 1, the amino acid sequence of the FR in the VH of a human antibody was selected. Kabat et al. classified the VHs of various existing human antibodies into three subgroups (HSG I to III) according to the homology of their amino acid sequences. Additionally, they reported consensus sequences in the each subgroup (Sequences of Proteins of Immunological Interest). Since it was suggested that the

immunogenicity of these consensus sequences might be more reduced in humans, it was determined to design the amino acid sequence of the VH of an anti-FGF-8 neutralizing CDR-grafted antibody on the basis of these consensus sequences. For preparing an anti-FGF-8 neutralizing CDR-grafted antibody with a higher activity, the amino acid sequence of an FR type with the highest homology to the amino acid sequence of the FR in the VH of KM1334 was selected for such designing, among the amino acid sequences of the FRs in the consensus sequences of the three VH subgroups of human antibodies. Table 1 showed the results of homology search. As shown in Table 1, the amino acid sequence of the FR in the VH region of KM1334 had the highest homology to the amino acid sequence thereof in subgroup I.

[Table 1]

Table 1

Homology between the amino acid sequences of the FRs in the consensus sequences of the each subgroup of human antibodies VH and the amino acid sequence of the FR in the VH of KM1334

HSG I	HSG II	HSG III
79.3%	51.7%	59.8%

Based on the foregoing results, the amino acid sequence of the CDR in the VH of the mouse anti-FGF-8 neutralizing antibody KM1334 was grafted in an appropriate position of the amino acid sequence of the FR in the consensus sequence of the VH subgroup I of human antibodies, to design the amino acid sequence HV.0 of the VH of the anti-FGF-8 neutralizing CDR-grafted antibody, as shown as SEQ ID No. 15.

Subsequently, the amino acid sequence of the VL of the anti-FGF-8 neutralizing CDR-grafted antibody was designed as follows. So as to graft the amino acid sequence of the CDR in the VL of the anti-FGF-8 neutralizing mouse antibody KM1334 as identified in 1(4) of Reference Example 1, the amino acid

sequence of the FR in the VL of a human antibody was selected. In the same manner as in the case of the VH, the amino acid sequence of FR with the highest homology to the amino acid sequence of the FR in the VL of KM1334 was selected among the amino acid sequences of the FRs in the consensus sequences of four VL subgroups of human antibodies. Table 2 showed the results of homology search. As shown in Table 2, the amino acid sequence of the FR in the VL of KM1334 had the highest homology to the amino acid sequence thereof in the subgroup II.

[Table 2]

Table 2

Homology between the amino acid sequences of the FRs in the consensus sequences of the subgroup of human antibodies VL and the amino acid sequence of the FR in the VL of KM1334

HSG I	HSG II	HSG III	HSG IV
66.3%	83.8%	66.2%	73.8%

Based on the foregoing results, the amino acid sequence of the CDR in the VL of the mouse anti-FGF-8 neutralizing antibody KM1334 was grafted in an appropriate position of the amino acid sequence of the FR in the consensus sequence of the VL subgroup II of human antibodies, to design the amino acid sequence LV.0 of the VL of the anti-FGF-8 neutralizing CDR-grafted antibody, as represented as SEQ ID No. 16.

The amino acid sequence HV.0 of the VH of the anti-FGF-8 neutralizing CDR-grafted antibody and the amino acid sequence LV.0 of the VL thereof, as designed above, are sequences in which only the amino acid sequence of the CDR in the mouse anti-FGF-8 neutralizing antibody KM1334 has been grafted in the amino acid sequence of the selected FR in the human antibody. In human CDR-grafted antibodies, generally, the binding activities thereof are reduced in many cases, when only the

amino acid sequence of the CDR of a mouse antibody is simply grafted in such antibodies. In order to avoid this, graft of an amino acid residue possibly influencing the activities among amino acid residues different in the FRs between a human antibody and a mouse antibody is done, together with the amino acid sequence of the CDR. In this Reference Example alike, it was examined to identify amino acid residues with a possible influence on the activity in the FR. First, the three-dimensional structure of the antibody V region (HV0LV0) comprising the amino acid sequence HV.0 of the VH and the amino acid sequence LV.0 of the VL of the anti-FGF-8 neutralizing CDR-grafted antibody as designed above was constructed by the computer modeling method. The three-dimensional structure coordinates were prepared using Software AbM (manufactured by Oxford Molecular), while the three-dimensional structure was displayed using Software Pro-Explore (manufactured by Oxford Molecular) or RasMol (manufactured by Glaxo), according to the attached instruction manuals. The computer model of the three-dimensional structure of the V region of the mouse anti-FGF-8 neutralizing antibody KM1334 was constructed in the same manner. Furthermore, amino acid residues different in the amino acid sequences of the FRs in the VH and VL of HV0LV0 from those of the amino acid sequences of the mouse anti-FGF-8 neutralizing antibody KM1334 were sequentially modified into amino acid residues at the corresponding positions in the mouse anti-FGF-8 neutralizing antibody KM1334. A three-dimensional model comprising the resulting amino acid sequence was constructed in the same manner. Then, the three-dimensional structures of the V region of the mouse anti-FGF-8 neutralizing antibody KM1334, HV0LV0 and the resulting modified product were compared to each other. Consequently, the following amino acid residues with possible influences on the antibody activity by modifying the three-dimensional structure of the antigen-binding site were selected among the amino acid residues in the FR in HV0LV0; Lys-12, Ala-13, Ala-40, Pro-41, Val-48, Val-68, Ile-70, Thr-74, Thr-76, Glu-82, Arg-84, Arg-87, and

Tyr-95 in HV.0, and in LV.0, Ile-2, Val-3, Gln-14, Gln-15, Gln-50, Leu-51, and Tyr-92. At least one or more among these selected amino acid residues were modified into the amino acid residues in the mouse antibody KM1334, to design the VH and VL of a human CDR-grafted antibody with various modifications.

(2) Construction of cDNA encoding the VH of the anti-FGF-8 neutralizing CDR-grafted antibody

cDNA encoding the amino acid sequence HV.0 of the VH in the anti-FGF-8 neutralizing CDR-grafted antibody as designed in Reference Example 2, 1(1) was constructed by PCR method as follows.

First, a secretory signal sequence in the H chain of the mouse anti-FGF-8 neutralizing antibody KM1334 as represented as SEQ ID No. 2 was conjugated to the designed amino acid sequence, to prepare a full-length amino acid sequence of the antibody. The resulting amino acid sequence was then converted to genetic codons. When plural genetic codons existed for one amino acid residue, the corresponding genetic codon was determined, taking account of the frequency in the nucleotide sequences of antibody genes (Sequences of Proteins of Immunological Interest). The determined genetic codons were connected together, to design the nucleotide sequence of the cDNA encoding the full-length amino acid sequence of the antibody V region. Additionally, sequences for binding amplification primers for PCR reaction (including restriction endonuclease recognition sequences for cloning into a vector for use in the expression of humanized antibodies) were added to the 5'- and 3'-termini of the resulting nucleotide sequence. The designed nucleotide sequence was divided into six nucleotide sequences of about 100 nucleotides, starting from the 5′ terminus (so that adjacent nucleotide sequences might have an overlapping sequence of about 20 nucleotides at each terminus). The sequences are shown in an alternate order of sense chain and anti-sense chain, practically as six synthetic oligonucleotides represented as SEQ ID Nos. 17 through 22 (manufactured by GENSET).

Each of the oligonucleotides was added to a final

concentration of 0.1 μmol/L to a reaction solution of 50 μL, for PCR reaction, using 0.5 μmol/L M13 primer RV (manufactured by Takara Shuzo), 0.5 μmol/L of M13 primer M4 (manufactured by Takara Shuzo) and 1 unit of KOD Polymerase (manufactured by TOYOBO) according to the attached instruction manual for the KOD Polymerase. The reaction conditions then were the conditions described in the instruction manual (30 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 74°C for 60 seconds). After the reaction solution was precipitated with ethanol, the resulting precipitate was dissolved in sterile water. The resulting solution reacted with 10 units of a restriction endonuclease HindIII (manufactured by Takara Shuzo) and 10 units of a restriction endonuclease NotI (manufactured by New England BioLabs), at 37°C for one hour. The reaction solution was fractionated by agarose gel electrophoresis, to recover about 0.3 μg of a HindIII-NotI fragment of about 0.47 kb.

Subsequently, 3 μg of plasmid pBluescript SK(-) (manufactured by Stratagene) reacted with 10 units of a restriction endonuclease HindIII and 10 units of a restriction endonuclease NotI at 37°C for one hour. The resulting reaction solution was fractionated by agarose gel electrophoresis, to recover about 2.9 μg of a HindIII-NotI fragment of about 2.95 kb.

Then, 0.1 μg of the HindIII-NotI fragment as a PCR product of the VH of the anti-FGF-8 neutralizing CDR-grafted antibody and 0.1 μg of the HindIII-NotI fragment of the plasmid pBluescript SK(-), as obtained above, were added to sterile water in a total amount of 10 μL, for ligation using Ligation High (manufactured by TOYOBO). The thus-obtained recombinant plasmid DNA solution was used to transform an Escherichia coli strain JM109. Then, each plasmid DNA was prepared from 10 clones of the resulting transformants. The nucleotide sequences were analyzed using Big Dye Terminator Kit Version 2 (manufactured by Applied Biosystems). As a result of the analysis of the nucleotide sequences, a plasmid phKM1334HV0 with the desired nucleotide sequence was obtained, as shown in Fig. 8.

Amino acid residues in the FR designed in Reference Example 2, 1(1) were modified as follows. For modification of the Lys-12 with Ala, for example, the oligonucleotide represented as SEQ ID No.23 was used in place of the oligonucleotide represented as SEQ ID No.18, for the same reaction as for the construction of the plasmid phKM1334HV0 described above, to obtain a plasmid phKM1334HV12 with the intended nucleotide sequence as shown in Fig. 9. The genetic codons for the amino acid residues after such modification were the genetic codons for the mouse antibody KM1334.

For modification of the Val-68 with Ala, for example, 50 ng of the plasmid phKM1334HV0 was used as template, while the synthetic DNA with the nucleotide sequence represented as SEQ ID No.24 for mutation induction (manufactured by GENSET) and M13 primer RV (manufactured by Takara Shuzo) were added as primers to each final concentration of 0.3 μmol/L, for PCR reaction in a 50-μl system, using KOD plus polymerase (manufactured by TOYOBO) according to the instruction manual attached, where first heating at 94°C for 2 minutes was followed by 30 cycles of a condition of 94°C for 15 seconds, 55°C for 30 seconds and 68°C for one minute. After the resulting reaction solution was precipitated with ethanol, the resulting precipitate was dissolved in sterile water, for reaction with 10 units of a restriction endonuclease SacII (manufactured by Takara Shuzo) at 37°C for one hour. The resulting reaction solution was fractionated by agarose gel electrophoresis, to recover about 0.3 μg of a SacII fragment of about 0.30 kb.

Then, 3 μg of the plasmid phKM1334HV0 reacted with 10 units of a restriction endonuclease SacII at 37°C for one hour. The resulting reaction solution was fractionated by agarose gel electrophoresis, to recover about 2.5 μg of a SacII-SacII fragment of about 3.04 kb.

Subsequently, 0.1 μg of the SacII-SacII fragment as a PCR product and 0.1 μg of the SacII-SacII fragment derived from the plasmid phKM1334HV0, as obtained above, were added to sterile water in a total amount of 10 μL, for ligation using Ligation

High (manufactured by TOYOBO). The thus-obtained recombinant plasmid DNA solution was used to transform an Escherichia coli strain JM109, and then, each plasmid DNA was prepared from 10 clones of the resulting transformants. The nucleotide sequences were analyzed using Big Dye Terminator Kit Version 2 (manufactured by Applied Biosystems). As a result of the analysis of the nucleotide sequences, a plasmid phKM1334HV68 with the desired nucleotide sequence, as shown in Fig. 10, was obtained. The genetic codons for the amino acid residues after such modification were the genetic codons observed for the mouse antibody KM1334.

(3) Construction of cDNA encoding the VL of the anti-FGF-8 neutralizing CDR-grafted antibody

cDNA encoding the amino acid sequence LV.0 of the VL of the anti-FGF-8 neutralizing CDR-grafted antibody as designed in Reference Example 2, 1(1) was constructed by PCR method in the same manner as for the VH, as follows. As the secretorysignal sequence, herein, the sequence of the L chain of the anti-FGF-8 neutralizing mouse antibody KM1334 as represented as SEQ ID No.4 was used.

First, six synthetic oligonucleotides with the nucleotide sequences represented as SEQ ID Nos. 25, 26, 27, 28, 29 and 30 were synthetically prepared (manufactured by GENSET). Each oligonucleotide was added to a reaction solution of 50 μL to give final concentration of 0.1 μmol/L, PCR reaction was progressed, using 0.5 μmol/L M13 primer RV (manufactured by Takara Shuzo), 0.5 μmol/L M13 primer M4 (manufactured by Takara Shuzo) and one unit of KOD polymerase (manufactured by TOYOBO) according to the attached instruction manual for the KOD polymerase. The reaction conditions then followed the conditions described in the instruction manual (30 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 74°C for 60 seconds). After the reaction solution was precipitated with ethanol, the resulting precipitate was dissolved in sterile water, for reaction at 37°C for one hour, using 10 units of a restriction endonuclease EcoRI (manufactured by Takara Shuzo) and 10 units

of a restriction endonuclease HindIII(manufactured by Takara Shuzo). The reaction solution was fractionated by agarose gel electrophoresis, to recover about 0.3 μg of an EcoRI-HindIII fragment of about 0.44 kb.

Further, 3 μg of the plasmid pBluescript SK(-) (manufactured by Stratagene) reacted with 10 units of restriction endonuclease EcoRI and 10 units of restriction endonuclease HindIII at 37°C for one hour. The reaction solution was fractionated by agarose gel electrophoresis, to recover about 2.9 μg of an EcoRI-HindIII fragment of about 2.95 kb.

Subsequently, 0.1 μg of the EcoRI-HindIII fragment as a PCR product of the VL in the anti-FGF-8 neutralizing CDR-grafted antibody and 0.1 μg of the EcoRI-HindIII fragment of the plasmid pBluescript SK(-), as obtained above, were added to sterile water in a total amount of 10 μL, for ligation using Ligation High (manufactured by TOYOBO). The thus-obtained recombinant plasmid DNA solution was used to transform an Escherichia coli strain JM109. From 10 clones of the resulting transformants were prepared each plasmid DNAs, for the analysis of the nucleotide sequences thereof, using Big Die Terminator Kit Version 2 (manufactured by Applied Biosystems). The analysis of the nucleotide sequences consequently showed that the plasmid phKM1334LV0 with the intended nucleotide sequence as shown in Fig. 11 was obtained.

Subsequently, amino acid residues in the FR designed in Reference Example 2, 1(1) were modified by any of the following methods. For modification of the Tyr-92 with Phe, the oligonucleotide represented as SEQ ID No.31 was used instead of the oligonucleotide represented as SEQ ID No. 29, for the same reaction as for the construction of the plasmid phKM1334LV0, so that the plasmid phKM1334LV92 with the intended nucleotide sequence as shown in Fig. 12 was obtained. The genetic codons for the amino acid residues after such modification were the genetic codons observed for the mouse antibody KM1334.

For modification of the Ile-2 with Val, for example, 50 ng of the plasmid phKM1334LV0 prepared in this section was used

as template, while a synthetic DNA with the nucleotide sequence represented as SEQ ID No. 32 for mutation induction (manufactured by GENSET) and M13 primer RV (manufactured by Takara Shuzo) were respectively added to give final concentrations of 0.3 μmol/L, for PCR reaction in a 50-μl system according to the attached instruction manual for KOD plus polymerase (manufactured by TOYOBO), under a condition that heating at 94°C for 2 minutes was followed by 30 cycles of a reaction at 94°C for 15 seconds, 55°C for 30 seconds and 68°C for one minute. After the reaction solution was precipitated with ethanol, the resulting precipitate was dissolved in sterile water, for a reaction at 37°C for one hour, using 10 units each of restriction endonucleases Tth111I (manufactured by Takara Shuzo) and EcoRI (manufactured by Takara Shuzo). The reaction solution was fractionated by agarose gel electrophoresis, to recover about 0.2 μg of an EcoRI-Tth111I fragment of about 0.08 kb.

Subsequently, 3 μg of the plasmid phKM1334LV0 was reacted with 10 units of a restriction endonuclease Tth111I and EcoRI at 37°C for one hour. The reaction solution was fractionated by agarose gel electrophoresis, to recover about 2.6 μg of a EcoRI-Tth111I fragment of about 3.42 kb.

Then, 0.1 μg of the EcoRI-Tth111I fragment derived from the PCR products and 0.1 μg of the EcoRI-Tth111I fragment derived from the plasmid phKM1334LV0 as obtained above were added to sterile water of 10 μl in total, for ligation using Ligation High (manufactured by TOYOBO). The recombinant plasmid DNA solution thus obtained was used to transform an Escherichia coli strain JM109. From 10 clones of the resulting transformants were prepared each plasmid DNAs, for the analysis of the nucleotide sequences thereof, using Big Dye Terminator Kit Version 2 (manufactured by Applied Biosystems). The analysis of the nucleotide sequences consequently showed that the plasmid phKM1334LV2 with the intended nucleotide sequence as shown in Fig. 13 was obtained. The genetic codons for the amino acid residues after such modification were the genetic codons observed for the mouse antibody KM1334.

(4) Construction of a vector expressing the anti-FGF-8 neutralizing CDR-grafted antibody

Using the vector pKANTEX93 for use in expressing humanized antibodies as described in WO 97/10354 and the plasmids phKM1334HV0 and phKM1334LV0 obtained in Reference Example 2, 1 (2) and (3), an expression vector pKANTEX1334HV0LV0 of the anti-FGF-8 neutralizing CDR-grafted antibody was constructed in the following manner.

3 μg of the plasmid phKM1334HV0 obtained in Reference Example 2, 1 (2) reacted with 10 units of a restriction endonuclease ApaI (manufactured by Takara Shuzo) and 10 units of a restriction endonuclease NotI (manufactured by New England BioLabs) at 37°C for one hour. The reaction solution was fractionated by agarose gel electrophoresis, to recover about 0.3 μg of an ApaI-NotI fragment of about 0.47 kb.

Then, 3 μg of the vector pKANTEX93 for use in expressing humanized antibodies reacted with 10 units of a restriction endonuclease ApaI and 10 units of a restriction endonuclease NotI at 37°C for one hour. The reaction solution was fractionated by agarose gel electrophoresis, to recover about 2 μg of an ApaI-NotI fragment of about 12.75 kb.

Then, 0.1 μg of the phKM1334HV0-derived NotI-ApaI fragment and 0.1 μg of the plasmid pKANTEX93-derived NotI-ApaI fragment as obtained above were added to sterile water in a total amount of 10 μL, for ligation using Ligation High (manufactured by TOYOBO). The thus-obtained recombinant plasmid DNA solution was used to transform an Escherichia coli strain JM109, to obtain the plasmid pKANTEX1334HV0 shown in Fig. 14.

Subsequently, 3 μg of the plasmid phKM1334LV0 obtained in Reference Example 2, 1(3) reacted with 10 units of a restriction endonuclease EcoRI (manufactured by Takara Shuzo) and 10 units of a restriction endonuclease BsiWI (manufactured by New England BioLabs) at 37°C for one hour. The reaction solution was fractionated by agarose gel electrophoresis, to recover about 0.3 μg of an EcoRI-BsiWI fragment of about 0.44

kb.

Then, 3 μg of the above-obtained plasmid pKANTEX1334HV0 was reacted with 10 units of a restriction endonuclease EcoRI and 10 units of a restriction endonuclease BsiWI at 37°C for one hour. The reaction solution was fractionated by agarose gel electrophoresis, to recover about 2 μg of an EcoRI-BsiWI fragment of about 13.20 kb.

Subsequently, 0.1 μg of the phKM1334LV0-derived EcoRI-BsiWI fragment and 0.1 μg of the plasmid pKANTEX1334HV0-derived EcoRI-BsiWI fragment as obtained above were added to sterile water in a total amount of 10 μL, for ligation using Ligation High (manufactured by TOYOBO). The thus-obtained recombinant plasmid DNA solution was used to transform an Escherichia coli strain JM109, to obtain the expression vector pKANTEX1334HV0LV0 shown in Fig. 14.

Using 400 ng of the resulting plasmid, the nucleotide sequence was analyzed by Big Dye Terminator Kit Version 2 (manufactured by Applied Biosystems) according to the dideoxy method. As a result, it was confirmed that a plasmid with the desired DNA cloned therein was obtained.

Expression vectors for the VH and the VL with modification of amino acid residues in the FRs therein were constructed in the same manner.

(5) Stable expression of the anti-FGF-8 neutralizing CDR-grafted antibodies in an animal cell

The stable expression of the anti-FGF-8 neutralizing CDR-grafted antibody in an animal cell was done by the method described in 2(2) of Reference Example 1.

(6) Assaying the activity of the anti-FGF-8 neutralizing CDR-grafted antibody to bind to FGF-8 (ELISA)

The activity of the anti-FGF-8 neutralizing CDR-grafted antibody to bind to FGF-8 was assayed by ELISA described above in 2(3) of Reference Example 1.

[Effect of the Invention]

The present invention provides an agent for preventing

or treating arthritis, a cartilage protecting agent, a joint destruction inhibitor and a synovial membrane growth inhibitor comprising an anti-FGF-8 neutralizing antibody as an active ingredient, as well as a diagnostic agent of arthritis comprising an anti-FGF-8 antibody as an active ingredient and a diagnostic method for judging arthritis using the antibody.

"Sequence Listing Free Text"

SEQ ID NO. 11 - Primer for amplifying VH of KM1334

SEQ ID NO. 12 - Primer for amplifying VH of KM1334

SEQ ID NO. 13 - Primer for amplifying VL of KM1334

SEQ ID NO. 14 - Primer for amplifying VH of KM1334

SEQ ID NO. 15 - Amino acid sequence, HV.0 designed for VH of an anti-FGF-8 neutralizing CDR-grafted antibody designed

SEQ ID NO. 16 - Amino acid sequence, HL.0 designed for VL of an anti-FGF-8 neutralizing CDR-grafted antibody designed

SEQ ID NO. 17 - Synthetic DNA for construction of DNA encoding HV.0

SEQ ID NO. 18 - Synthetic DNA for construction of DNA encoding HV.0

SEQ ID NO. 19 - Synthetic DNA for construction of DNA encoding HV.0

SEQ ID NO. 20 - Synthetic DNA for construction of DNA encoding HV.0

SEQ ID NO. 21 - Synthetic DNA for construction of DNA encoding HV.0

SEQ ID NO. 22 - Synthetic DNA for construction of DNA encoding HV.0

SEQ ID NO. 23 - Synthetic DNA for construction of DNA encoding HV.0 in which Lys 12 is modified to Ala

SEQ ID NO. 24 - Synthetic DNA for construction of DNA encoding HV.0 in which Val 68 is modified to Ala

SEQ ID NO. 25 - Synthetic DNA for production of DNA encoding HL.0

SEQ ID NO. 26 - Synthetic DNA for construction of DNA encoding HL.0

SEQ ID NO. 27 - Synthetic DNA for construction of DNA encoding HL.0

SEQ ID NO. 28- Synthetic DNA for construction of DNA encoding HL.0

SEQ ID NO. 29 - Synthetic DNA for construction of DNA encoding HL.0

SEQ ID NO. 30 - Synthetic DNA for construction of DNA encoding HL.0

SEQ ID NO. 31 - Synthetic DNA for construction of DNA encoding HL.0 in which Tyr 93 is modified to Phe

SEQ ID NO. 32 - Synthetic DNA for construction of DNA encoding HL.0 in which Ile 2 is modified to Val

[SEQUENCE LISTING]

SEQUENCE LISTING

<110> KYOWA HAKKO KOGYO CO., LTD

<120> Diagnostic agent and therapeutic agent for arthritis

<130> H13-2344S3

<160> 32

<170> PatentIn Ver. 2.1

<210> 1
<211> 420
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(420)

<400> 1
atg gaa tgg atc tgg atc ttt ctc ttc ttc ctc tca gga act aca ggt 48
Met Glu Trp Ile Trp Ile Phe Leu Phe Phe Leu Ser Gly Thr Thr Gly
1 5 10 15

gtc tac tcc cag gtt cag ctg cag cag tct gga gct gag gtg gcg agg 96
Val Tyr Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Val Ala Arg
20 25 30

ccc ggg gct tca gtg aaa ctg tcc tgc aag gct tct ggc tac acc ttc 144
Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

act gac tac tat cta aac tgg gtg aag cag agg tct gga cag ggc ctt 192
Thr Asp Tyr Tyr Leu Asn Trp Val Lys Gln Arg Ser Gly Gln Gly Leu
50 55 60

gag tgg att gga gag att gat cct gga agt gat agt ata tat tat aat 240

Glu Trp Ile Gly Glu Ile Asp Pro Gly Ser Asp Ser Ile Tyr Tyr Asn
65 70 75 80

gaa aac ttg gag ggc agg gcc aca ctg act gca gac aaa tcc tcc agc 288
Glu Asn Leu Glu Gly Arg Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
85 90 95

aca gcc tac atg cag ctc aac agc ctg aca tct gag gac tct gca gtc 336
Thr Ala Tyr Met Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val
100 105 110

tat ttc tgt gca aga tat ggg tat tct aga tac gac gta agg ttt gtc 384
Tyr Phe Cys Ala Arg Tyr Gly Tyr Ser Arg Tyr Asp Val Arg Phe Val
115 120 125

tac tgg ggc caa ggg act ctg gtc act gtc tct aca 420
Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Thr
130 135 140

<210> 2
<211> 140
<212> PRT
<213> Mus musculus

<400> 2
Met Glu Trp Ile Trp Ile Phe Leu Phe Phe Leu Ser Gly Thr Thr Gly
1 5 10 15

Val Tyr Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Val Ala Arg
20 25 30

Pro Gly Ala Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

Thr Asp Tyr Tyr Leu Asn Trp Val Lys Gln Arg Ser Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Glu Ile Asp Pro Gly Ser Asp Ser Ile Tyr Tyr Asn
65 70 75 80

Glu Asn Leu Glu Gly Arg Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
85 90 95

Thr Ala Tyr Met Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val
100 105 110

Tyr Phe Cys Ala Arg Tyr Gly Tyr Ser Arg Tyr Asp Val Arg Phe Val
115 120 125

Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Thr
130 135 140

<210> 3
<211> 393
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)..(393)

<400> 3
atg aag ttg cct gtt agg ctg ttg gtg ctg atg ttc tgg att cct gct 48
Met Lys Leu Pro Val Arg Leu Leu Val Leu Met Phe Trp Ile Pro Ala
1 5 10 15

tcc agg agt gat gtt ttg atg acc caa act cca ctc tcc ctg cct gtc 96
Ser Arg Ser Asp Val Leu Met Thr Gln Thr Pro Leu Ser Leu Pro Val
20 25 30

agt ctt gga gat caa gcc tcc atc tct tgc aga tct agt cag agt ctt 144
Ser Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu
35 40 45

gta cat agt aat gga aga acc tat tta gaa tgg tac ctg cag aaa cct 192
Val His Ser Asn Gly Arg Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro
50 55 60

ggc cag tca cca aag gtc ctg atc tac aaa gtt tcc aac cga att tct 240
Gly Gln Ser Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Ile Ser
65 70 75 80

ggg gtc cca gac agg ttc agt ggc agt gga tca ggg aca gat ttc aca 288
Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
85 90 95

ctc aaa atc agc aga gtg gag gct gag gat ctg gga gtt tat ttc tgc 336
Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys
100 105 110

ttt cag ggt tca cat gtt ccg tac acg ttc gga ggg ggg acc aag ctg 384
Phe Gln Gly Ser His Val Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu
115 120 125

gaa ata aaa 393
Glu Ile Lys
130

<210> 4
<211> 131
<212> PRT
<213> Mus musculus

<400> 4
Met Lys Leu Pro Val Arg Leu Leu Val Leu Met Phe Trp Ile Pro Ala
1 5 10 15

Ser Arg Ser Asp Val Leu Met Thr Gln Thr Pro Leu Ser Leu Pro Val

20 25 30

Ser Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu
35 40 45

Val His Ser Asn Gly Arg Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro
50 55 60

Gly Gln Ser Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Ile Ser
65 70 75 80

Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
85 90 95

Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys
100 105 110

Phe Gln Gly Ser His Val Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu
115 120 125

Glu Ile Lys
130

<210> 5
<211> 5
<212> PRT
<213> Mus musculus

<400> 5
Asp Tyr Tyr Leu Asn
1 5

<210> 6
<211> 17
<212> PRT
<213> Mus musculus

<400> 6
Glu Ile Asp Pro Gly Ser Asp Ser Ile Tyr Tyr Asn Glu Asn Leu Glu

1 5 10 15

Gly

<210> 7
<211> 12
<212> PRT
<213> Mus musculus

<400> 7
Tyr Gly Tyr Ser Arg Tyr Asp Val Arg Phe Val Tyr
1 5 10

<210> 8
<211> 16
<212> PRT
<213> Mus musculus

<400> 8
Arg Ser Ser Gln Ser Leu Val His Ser Asn Gly Arg Thr Tyr Leu Glu
1 5 10 15

<210> 9
<211> 7
<212> PRT
<213> Mus musculus

<400> 9
Lys Val Ser Asn Arg Ile Ser
1 5

<210> 10
<211> 9
<212> PRT
<213> Mus musculus

<400> 10
Phe Gln Gly Ser His Val Pro Tyr Thr
1 5

<210> 11
<211> 22

<212> DNA
<213> Artificial Sequence

<220>
<223> a primer for amplification of VH of KM1334

<400> 11
ctgaattcgc ggccgctagt cc 22

<210> 12
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> a primer for amplification of VH of KM1334

<400> 12
atgggccctt ggtggaggct gtagagacag tgaccagag 39

<210> 13
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> a primer for amplification of VL of KM1334

<400> 13
ctgaattcgc ggccgctgct gt 22

<210> 14
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> a primer for amplification of VL of KM1334

<400> 14
atcgtacgtt ttatttccag cttggtcc 28

<210> 15
<211> 121

<212> PRT
<213> Artificial Sequence

<220>
<223> HV.0, a designed amino acid sequence of VH of an anti-FGF-8 CDR-gr
afted neutralizing antibody

<400> 15
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr
20 25 30

Tyr Leu Asn Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45

Gly Glu Ile Asp Pro Gly Ser Asp Ser Ile Tyr Tyr Asn Glu Asn Leu
50 55 60

Glu Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Tyr Gly Tyr Ser Arg Tyr Asp Val Arg Phe Val Tyr Trp Gly
100 105 110

Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

<210> 16
<211> 112
<212> PRT
<213> Artificial Sequence

<220>
<223> HL.0, a designed amino acid sequence of VL of an anti-FGF-8

CDR-gr
afted neutralizing antibody

<400> 16
Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Pro Gly
1 5 10 15

Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
20 25 30

Asn Gly Arg Thr Tyr Leu Glu Trp Tyr Leu Gln Lys Pro Gly Gln Ser
35 40 45

Pro Gln Leu Leu Ile Tyr Lys Val Ser Asn Arg Ile Ser Gly Val Pro
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Phe Gln Gly
85 90 95

Ser His Val Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105 110

<210> 17
<211> 90
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HV.0

<400> 17
gttttcccag tcacgacgcg gccgcaccat ggaatggatc tggatctttc tcttcttcct 60

ctcaggaact acaggtgtct actcccaggt 90

<210> 18
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HV.0

<400> 18
aaggtgtatc cagatgcctt gcaggaaacc ttcactgagg ccccaggctt cttcacctca 60

gccccagact gcaccagctg cacctgggag tagacacct 99

<210> 19
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HV.0

<400> 19
ggcatctgga tacaccttca ccgactacta tctaaactgg gtgcgacagg cccctggaca 60

agggcttgag tggatgggag agattgatcc tggaagtga 99

<210> 20
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HV.0

<400> 20
agctccatgt aggctgtgct cgtggacgtg tccgcggtaa tggtgactct gccctccaag 60

ttttcattat aatatatact atcacttcca ggatcaatc 99

<210> 21
<211> 99
<212> DNA

<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HV.0

<400> 21
cacagcctac atggagctga gcagcctgag atctgaggac acggccgtgt attactgtgc 60

gagatatggg tattctagat acgacgtaag gtttgtcta 99

<210> 22
<211> 92
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HV.0

<400> 22
caggaaacag ctatgacaag cttgggccct tggtggaggc tgaggagacg gtgaccaggg 60

ttccctggcc ccagtagaca aaccttacgt cg 92

<210> 23
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HV.0 in which L
ys12 was mutated to Ala

<400> 23
aaggtgtatc cagatgcctt gcaggaaacc ttcactgagg ccccaggctt cgccacctca 60

gccccagact gcaccagctg cacctgggag tagacacct 99

<210> 24
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HV.0 in which V
al68 was mutated to Ala

<400> 24
tgtccgcggt aatggtggct 20

<210> 25
<211> 84
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HL.0

<400> 25
gttttcccag tcacgacgaa ttcacatgaa gttgcctgtt aggctgttgg tgctgatgtt 60

ctggattcct gcttccagat ccga 84

<210> 26
<211> 93
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HL.0

<400> 26
ctagatctgc aggagatgga ggccggctct ccaggggtga cgggcaggga gagtggagac 60

tgagtcatca caatatcgga tctggaagca gga 93

<210> 27
<211> 93
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HL.0

<400> 27
catctcctgc agatctagtc agagtcttgt acatagtaat ggaagaacct atttagaatg 60

gtacctgcag aagccagggc agtctccaca gct 93

<210> 28
<211> 93
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HL.0

<400> 28
aaatctgtgc ctgatccact gccactgaac ctgtcaggga ccccagaaat tcggttggaa 60

actttataga tcaggagctg tggagactgc cct 93

<210> 29
<211> 93
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HL.0

<400> 29
tggatcaggc acagatttta cactgaaaat cagcagagtg gaggctgagg atgttggggt 60

ttattactgc tttcagggtt cacatgttcc gta 93

<210> 30
<211> 81
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HL.0

<400> 30
caggaaacag ctatgacaag cttcgtacgt ttgatttcca ccttggtccc ttggccgaac 60

gtgtacggaa catgtgaacc c 81

<210> 31
<211> 93
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HL.0 in which T
yr93 was mutated to Phe

<400> 31
tggatcaggc acagatttta cactgaaaat cagcagagtg gaggctgagg atgttggggt 60

ttatttctgc tttcagggtt cacatgttcc gta 93

<210> 32
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> a synthetic DNA for construction of a DNA encoding HL.0 in which I
le2 was mutated to Val

<400> 32
gagactgagt catcacaaca tc 22

[Brief Description of the Drawings]

[Fig. 1] Fig. 1 is a graph showing a degradation activity of extracellular matrix of rabbit chondrocytes by FGF-8. The ordinate represents an amount of glycosaminoglycan remaining in extracellular matrix, and the abscissa represents the concentration (ng/mL) of FGF-8. The values represent the mean values ± the standard deviation (SE), and *** indicates $P<0.001$ (compared to a non-stimulated group, Dunnett test).

[Fig. 2] Fig. 2 is a graph showing inhibitory activity of antibody to degradation of extracellular matrix of rabbit chondrocytes with FGF-8. The ordinate represents an amount of glycosaminoglycan remaining in extracellular matrix, and the abscissa represents the concentration (μg/mL) of KM1334. The values represent the mean values ± the standard deviation (SE), and ### indicates $P<0.001$ (compared to a non-stimulated group, Student's t-test), ** indicates $P<0.01$ and *** indicates $P<0.001$ (compared to group added with 100ng/mL of FGF-8, Dunnett test).

[Fig. 3] Fig. 3 is a graph showing activity of promoting growth of rabbit synovial cells with FGF-8. The ordinate represents radioactivity of [^{3}H]thymidine incorporated into rabbit synovial cells, and the abscissa represents a concentration (ng/mL) of FGF-8. The values represent the mean values ± the standard deviation (SE), and * indicates $P<0.05$ (compared to a non-stimulated group, Steel test).

[Fig. 4] Fig. 4 is a graph showing inhibitory activity of antibody to promotion of growth of rabbit synovial cells with FGF-8. The ordinate represents radioactivity of [^{3}H]thymidine incorporated into rabbit synovial cells, and the abscissa represents a concentration (μg/mL) of KM1334. The values represent the mean values ± the standard deviation (SE), and ### indicates $P<0.001$ (compared to a group added with 100ng/mL of FGF-8, Student's t-test) and *** indicates $P<0.001$ (compared to a 0 group, Dunnett test).

[Fig. 5] Fig. 5 is a graph showing activity of promoting growth of human synovial cells with FGF-8. The ordinate

represents radioactivity of [^{3}H]thymidine incorporated into human synovial cells, and the abscissa represents a concentration (ng/mL)of FGF-8. The values represent the mean values ± the standard deviation (SE), and *** indicates P<0.001 (compared to a non-stimulated group, Dunnett test).

[Fig. 6] Fig. 6 is a graph showing inhibitory activity of the the antibody to promotion of growth of human synovial cells with FGF-8. The ordinate represents radioactivity of [^{3}H]thymidine incorporated into human synovial cells, and the abscissa represents a concentration (μg/mL) of KM1334. The values represent the mean values ± the standard deviation (SE), and ### indicates P<0.001 (compared to a non-stimulated group, Aspin-Welch test) and * indicates P<0.05 (compared to a group added with 500ng/mL of FGF-8, Steel test).

[Fig. 7] Fig. 7 is a flow chart showing construction of plasmid pKANTEX1334H and plasmid pKANTEX1334.

[Fig. 8] Fig. 8 is a flow chart showing construction of plasmid phKM1334HV0.

[Fig. 9] Fig. 9 is a flow chart showing construction of plasmid phKM1334HV12. * indicates the position of mutated gene for modifying the amino acid residue.

[Fig. 10] Fig. 10 is a flow chart showing construction of plasmid phKM1334HV68. * indicates the position of mutated gene for modifying the amino acid residue.

[Fig. 11] Fig. 11 is a flow chart showing construction of plasmid phKM1334LV0. * indicates the position of mutated gene for modifying the amino acid residue.

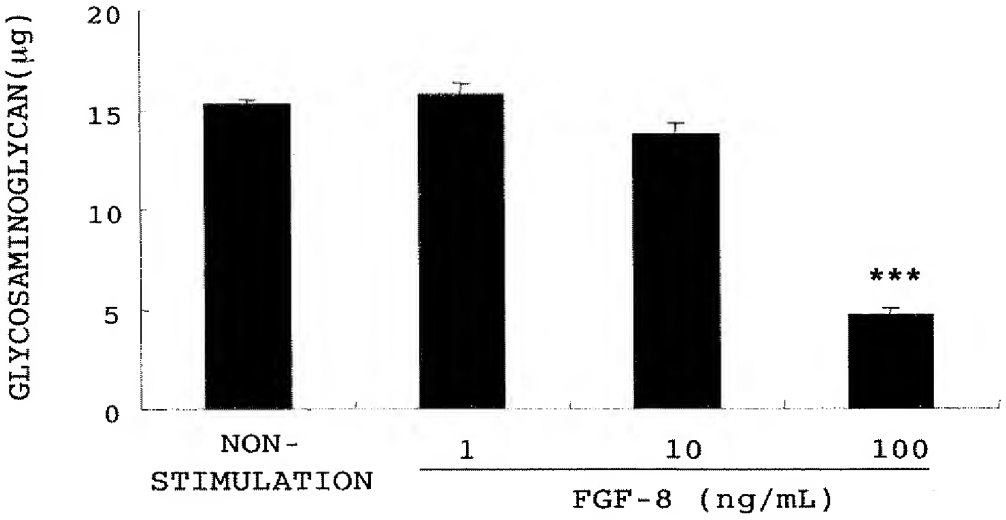
[Fig. 12] Fig. 12 is a flow chart showing construction of plasmid phKM1334LV92. * indicates the position of mutated gene for modifying the amino acid residue.

[Fig. 13] Fig. 13 is a flow chart showing construction of plasmid phKM1334LV2. * indicates the position of mutated gene for modifying the amino acid residue.

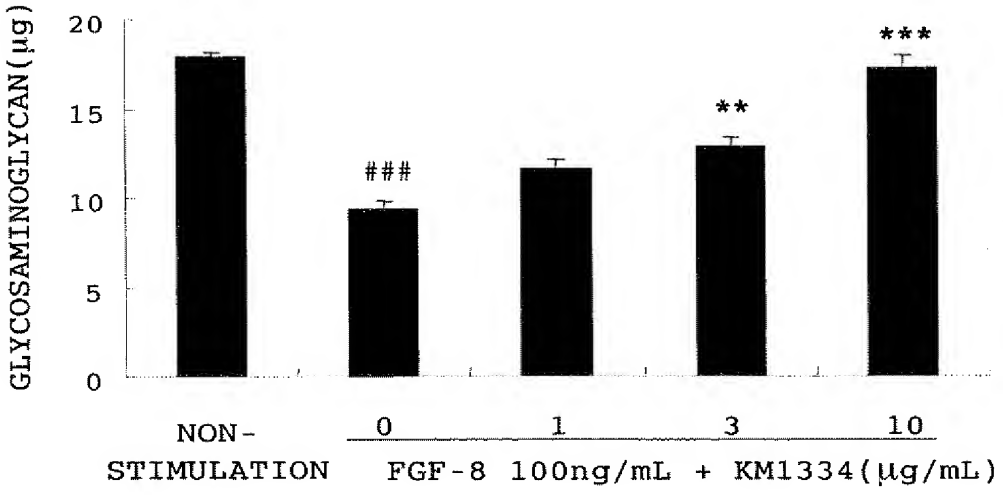
[Fig. 14] Fig. 14 is a flow chart showing construction of plasmid pKANTEX1334HV0 and plasmid pKANTEX1334HV0LV0.

[Name of Document] Drawings

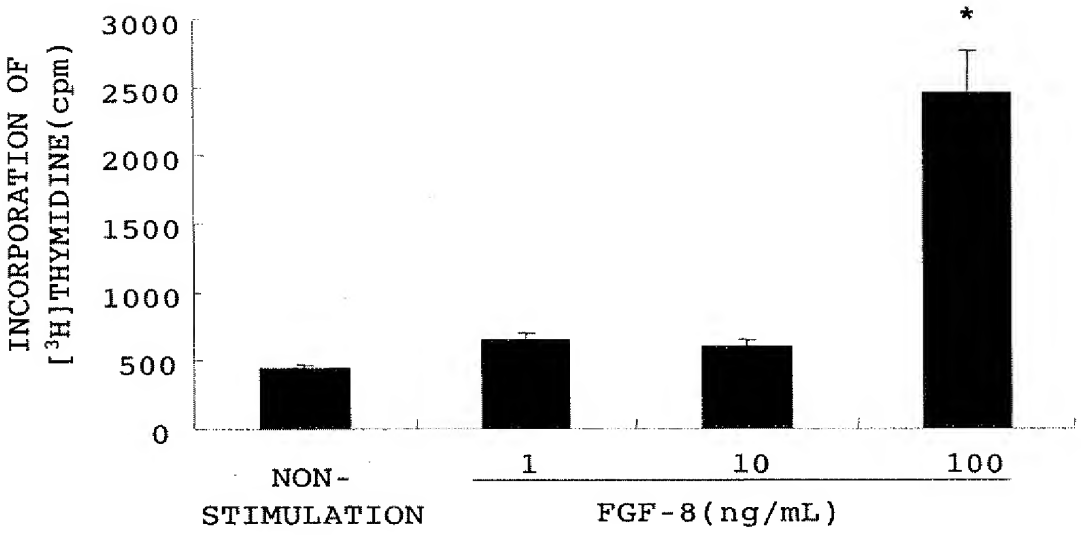
[Figure 1]



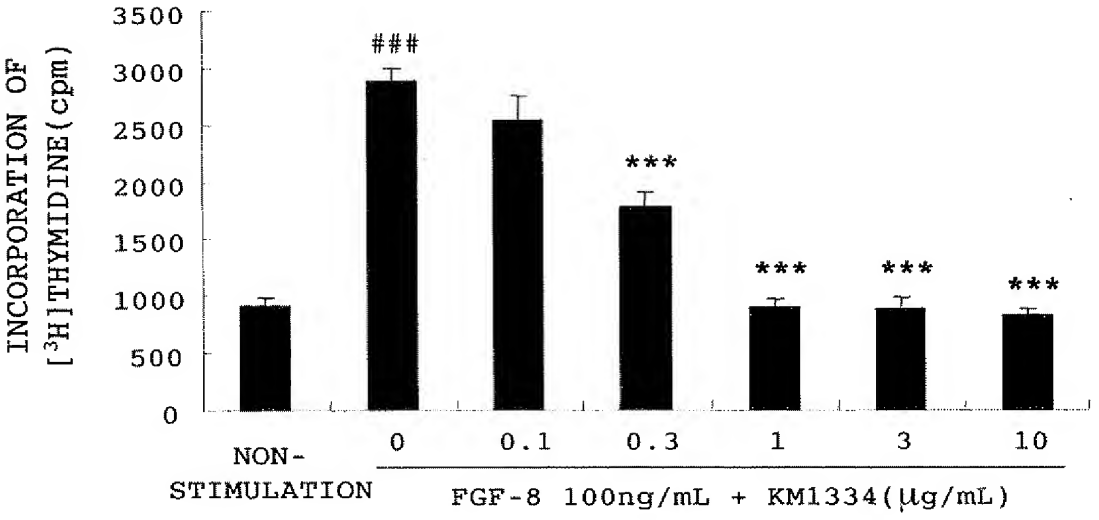
[Figure 2]



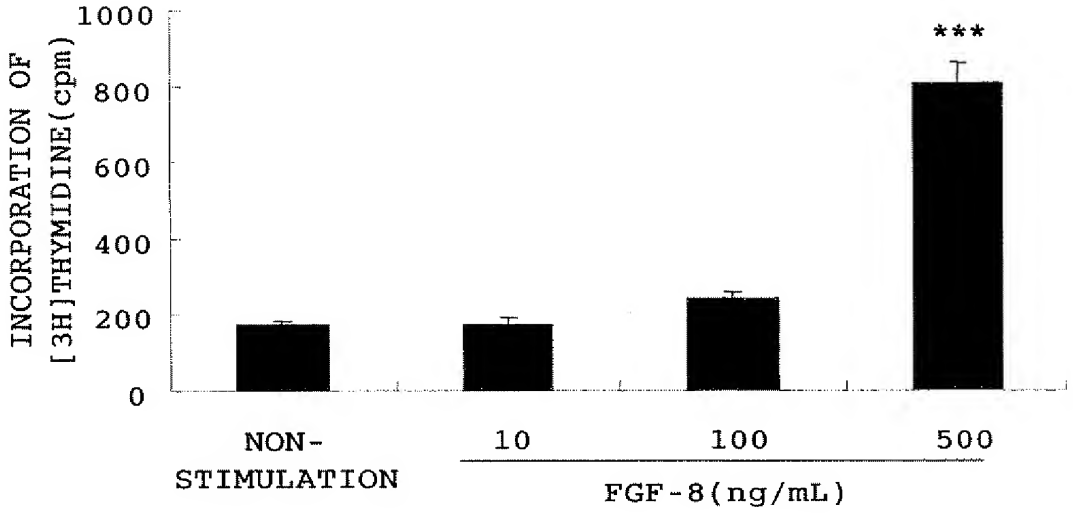
[Figure 3]



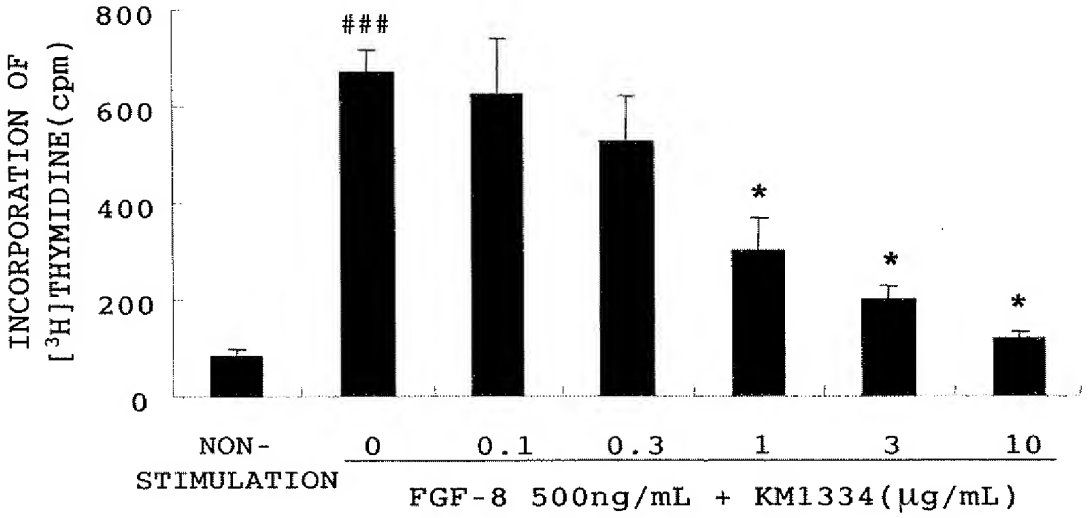
[Figure 4]



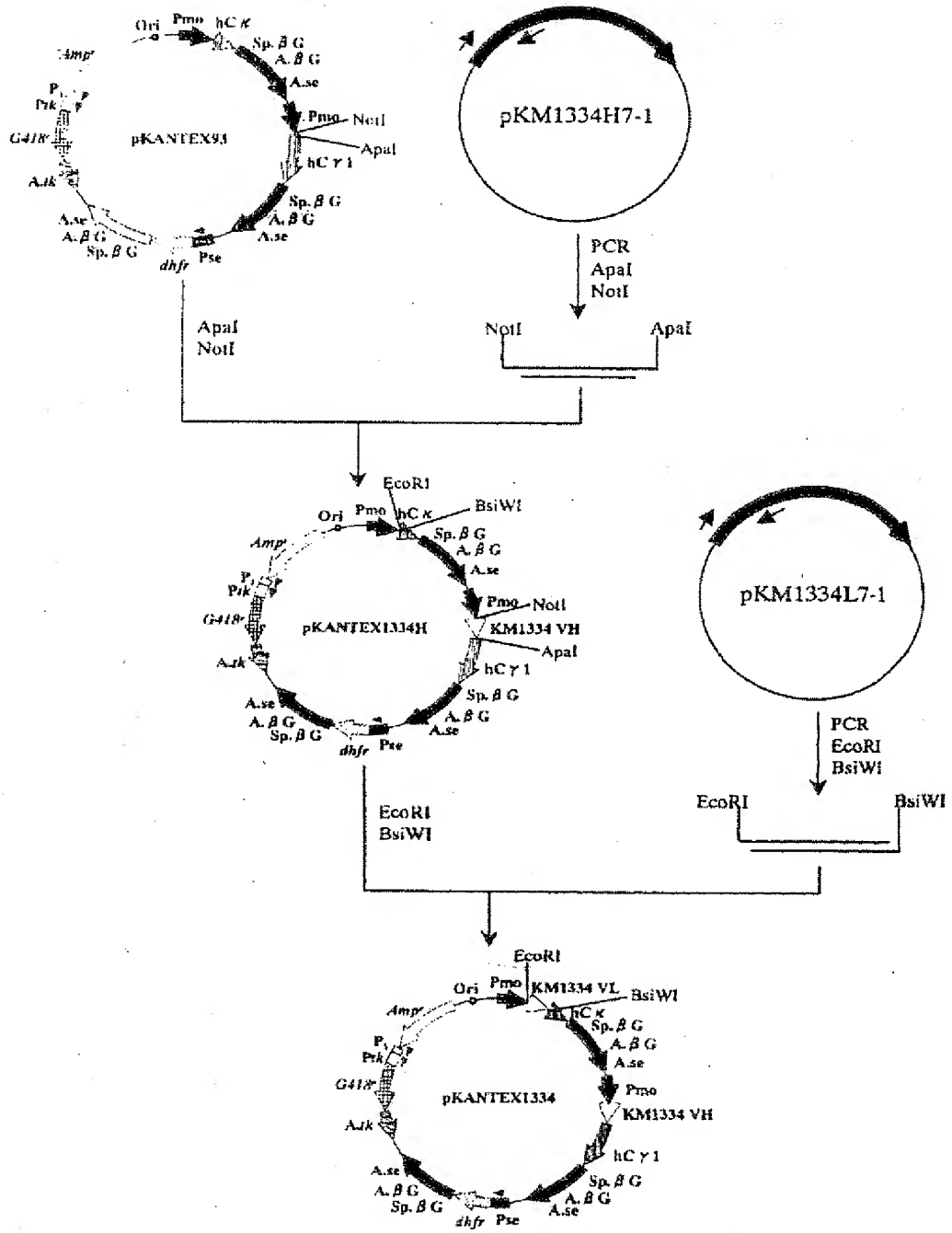
[Figure 5]



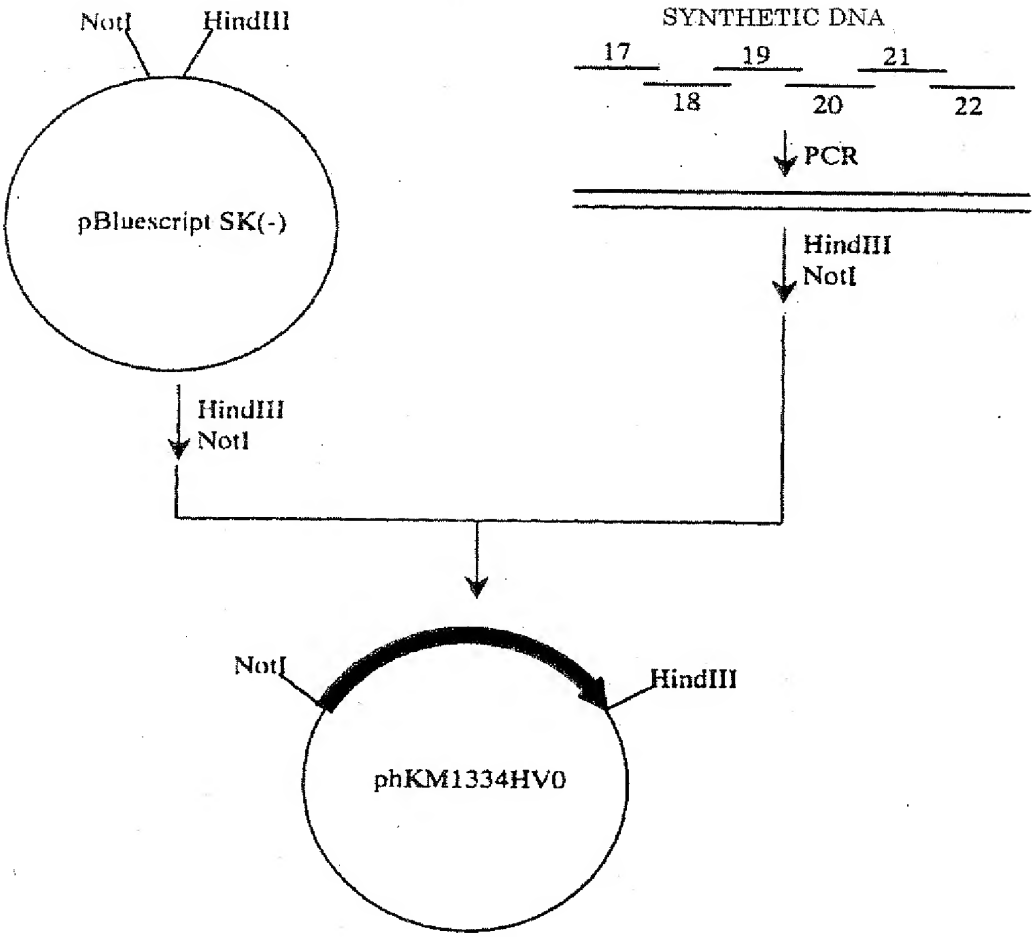
[Figure 6]



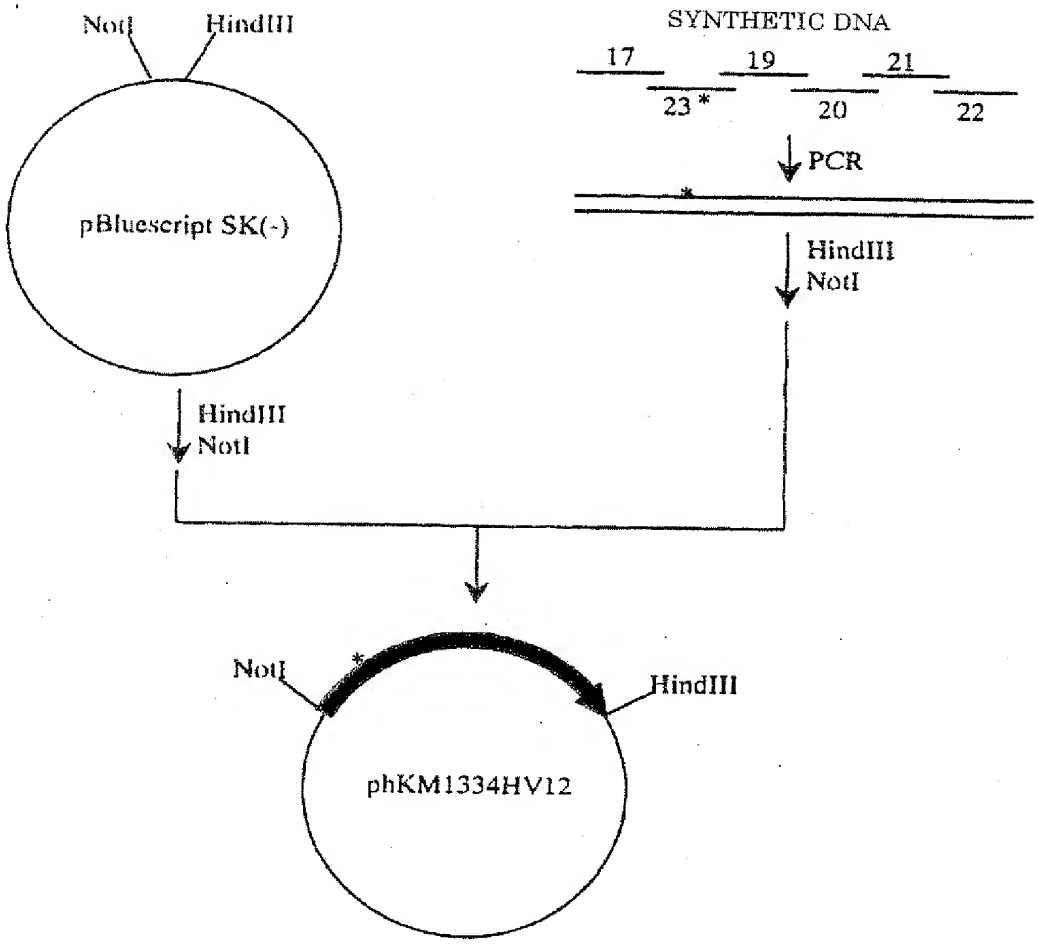
[Figure 7]


pKM1334H7-1
PCR
ApaI
NotI
NotI
ApaI
ApaI
NotI
pKANTEX93
Ori
Pmo
hC κ
Sp. β G
A. β G
A.se
Pmo
NotI
ApaI
hC γ 1
Sp. β G
A. β G
A.se
Sp. β G
A. β G
A.se
dhfr
Pse
Amp
G418
A.tk
EcoRI
BsiWI
pKM1334L7-1
PCR
EcoRI
BsiWI
EcoRI
BsiWI
pKANTEX1334H
KM1334 VH
EcoRI
BsiWI
KM1334 VL
pKANTEX1334
KM1334 VH

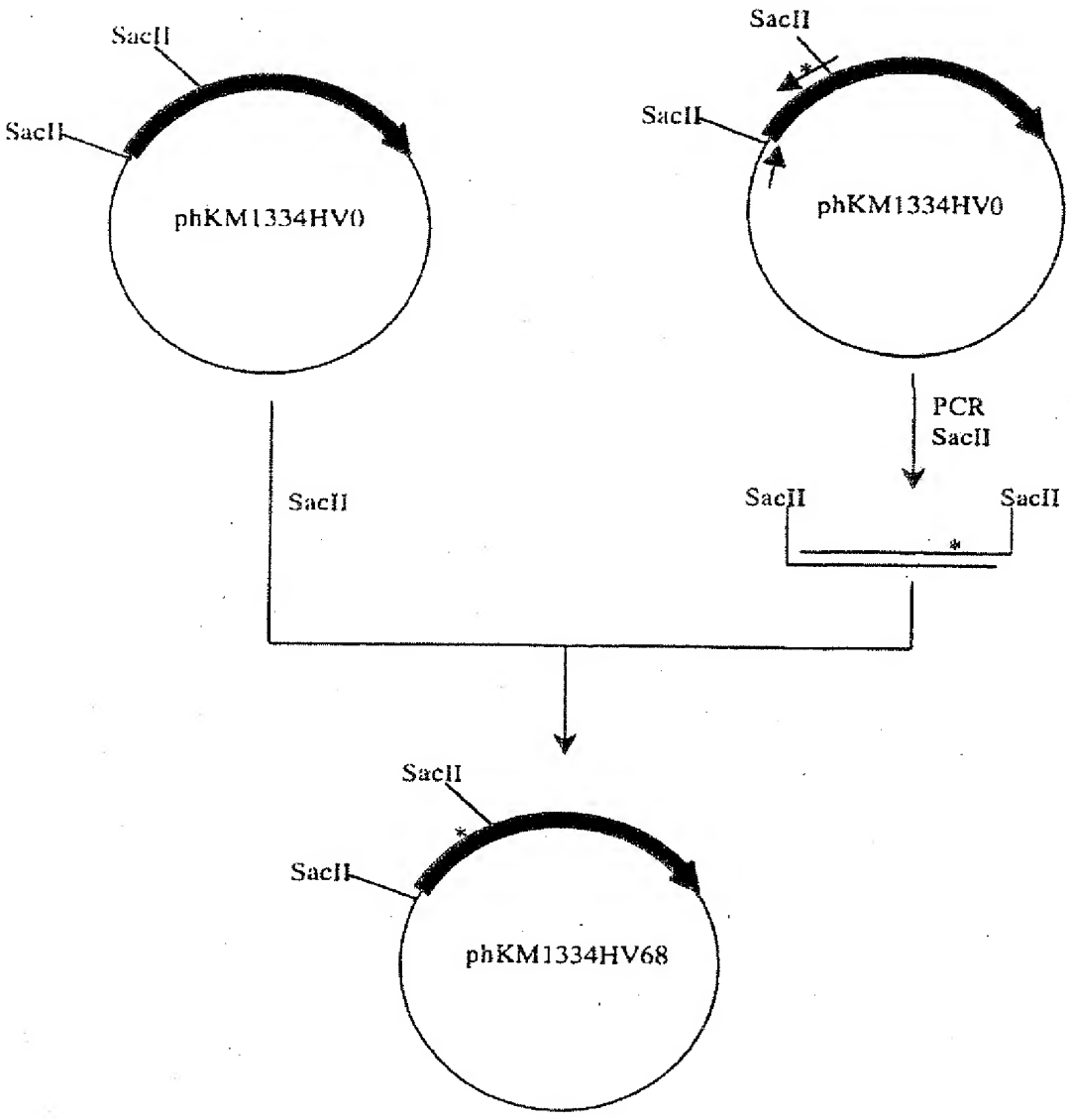
[Figure 8]


NotI
HindIII
pBluescript SK(-)
HindIII
NotI
SYNTHETIC DNA
17
19
21
18
20
22
PCR
HindIII
NotI
NotI
HindIII
phKM1334HV0

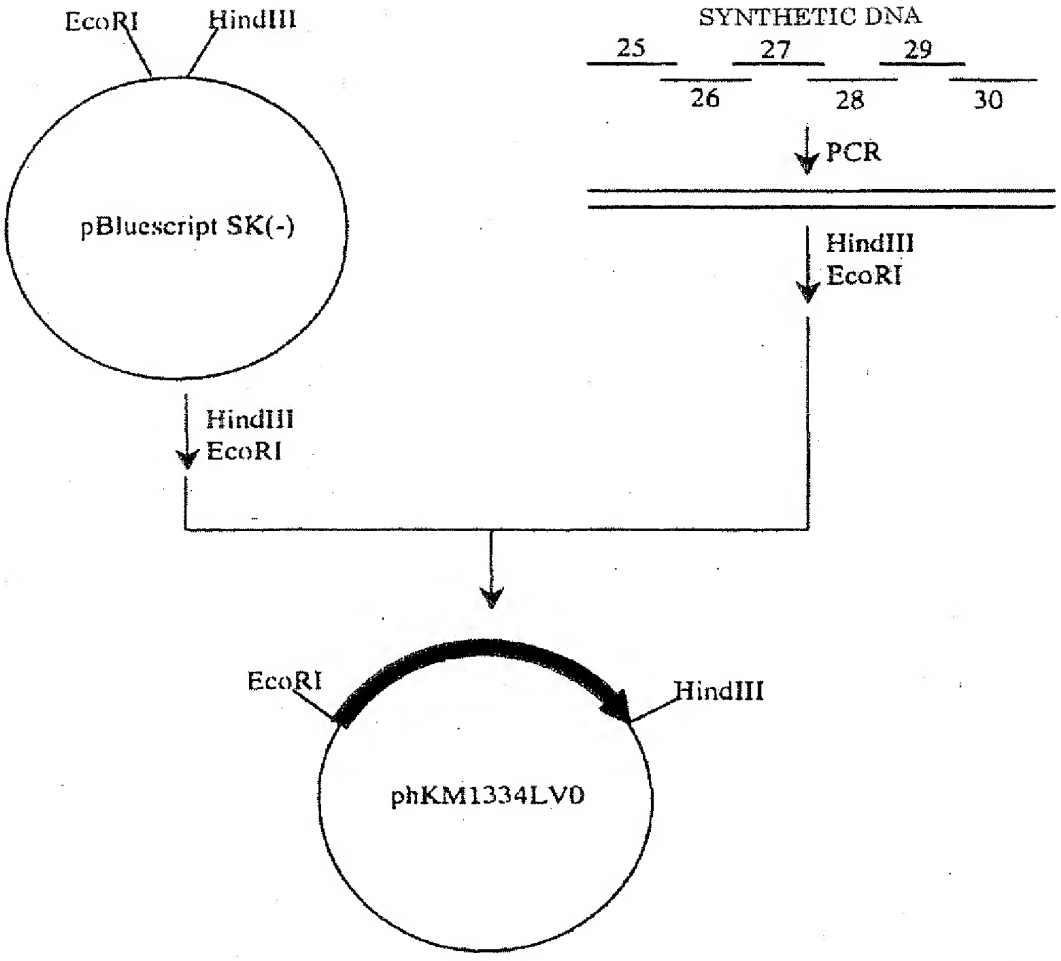
[Figure 9]


NotI
HindIII
pBluescript SK(-)
HindIII
NotI
SYNTHETIC DNA
17
19
21
23 *
20
22
PCR
HindIII
NotI
NotI
HindIII
phKM1334HV12

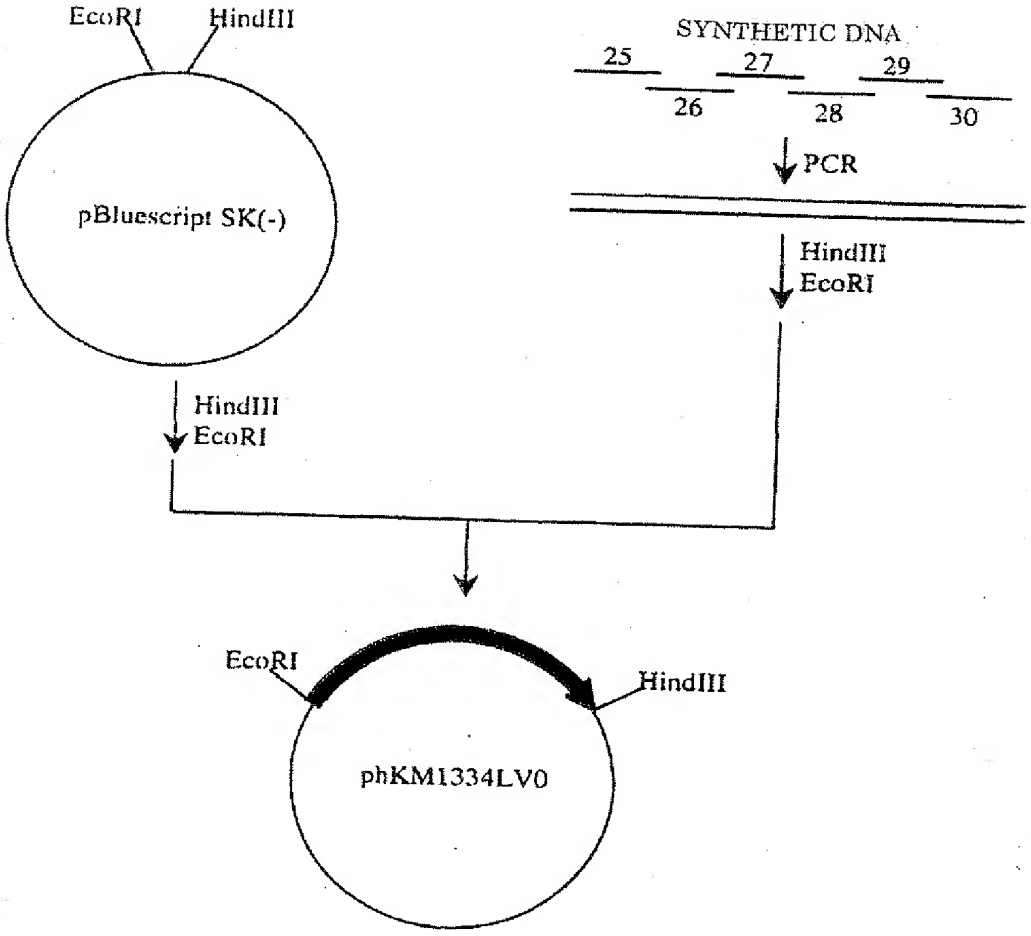
[Figure 10]


SacII
SacII
phKM1334HV0
SacII
SacII
phKM1334HV0
PCR
SacII
SacII
SacII
SacII
SacII
phKM1334HV68

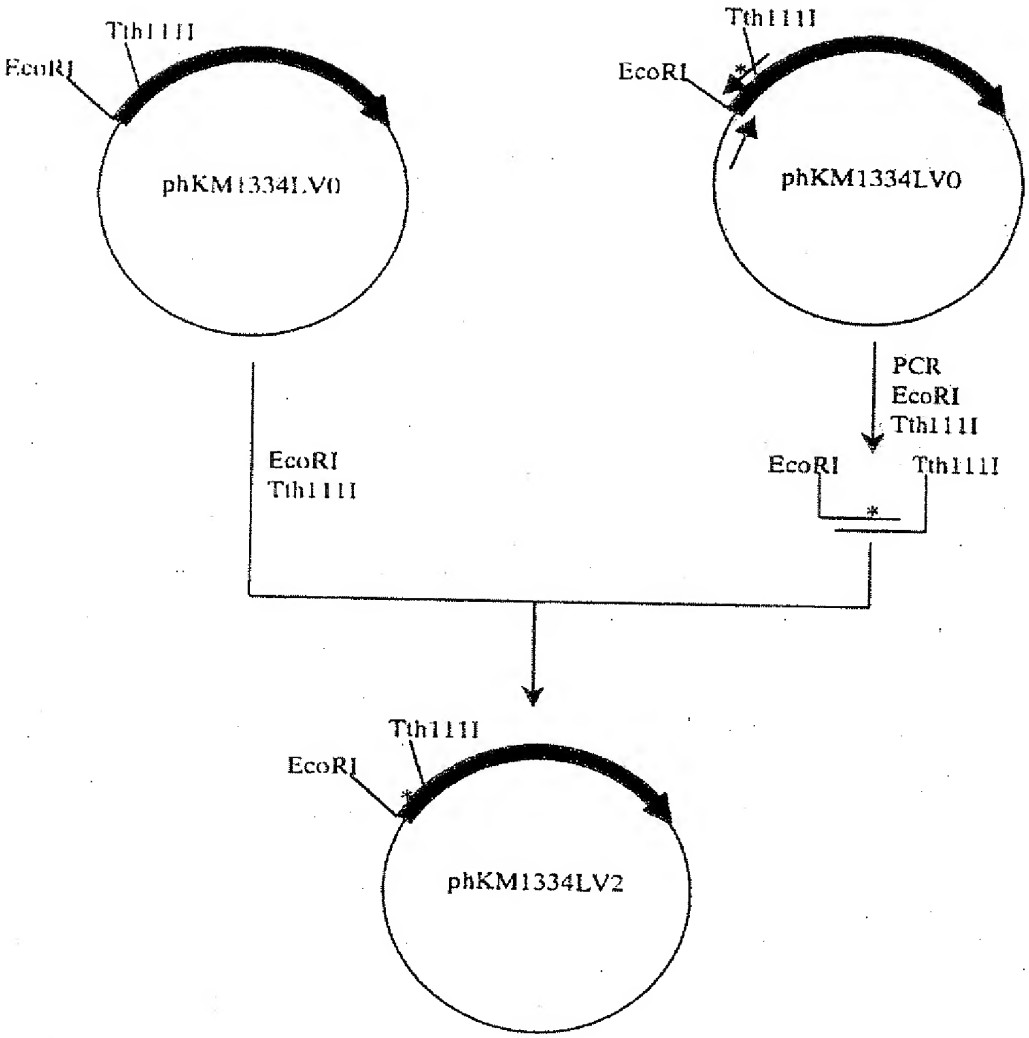
[Figure 11]


EcoRI
HindIII
pBluescript SK(-)
HindIII
EcoRI
SYNTHETIC DNA
25
27
29
26
28
30
PCR
HindIII
EcoRI
EcoRI
HindIII
phKM1334LV0

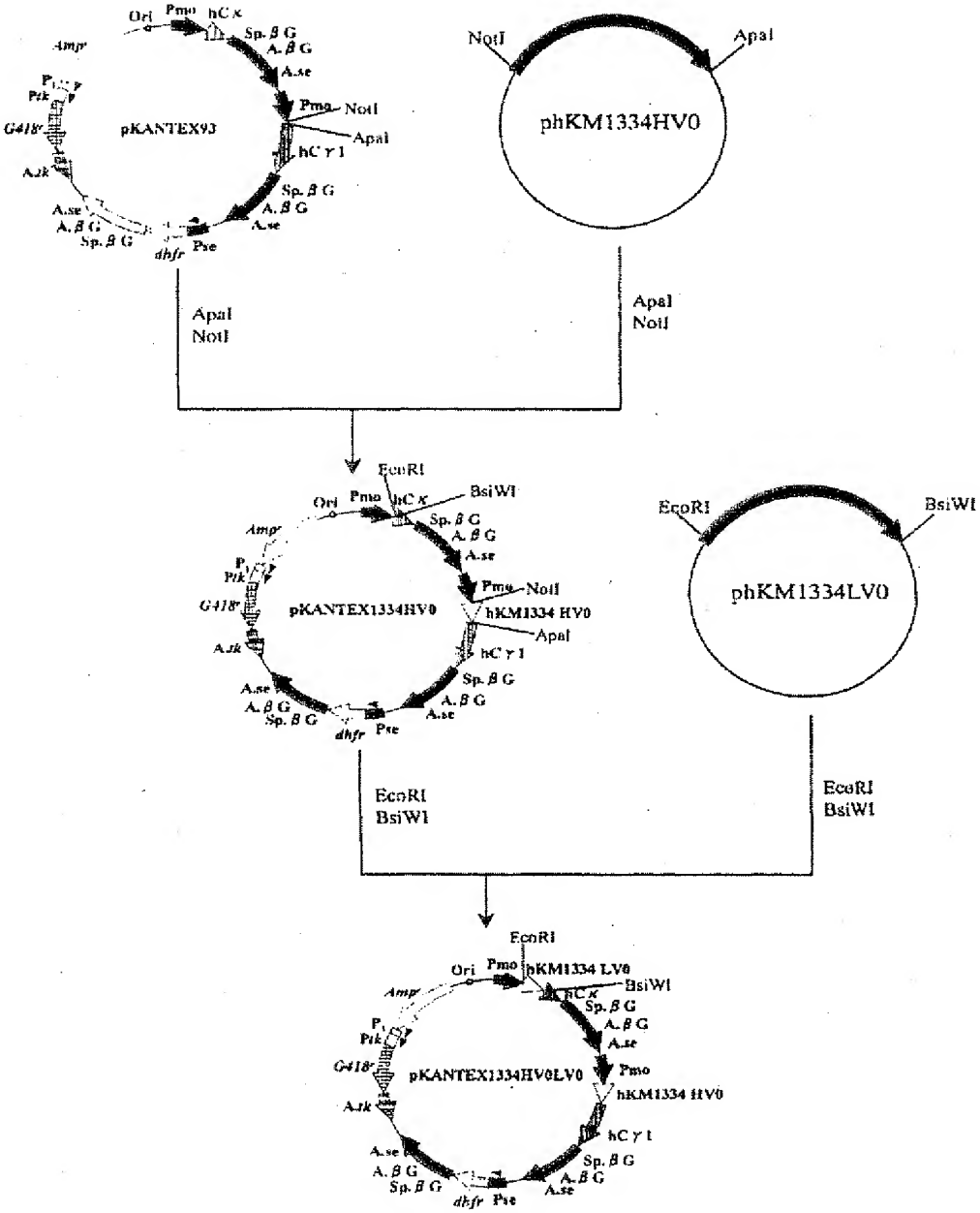
[Figure 12]


EcoRI
HindIII
pBluescript SK(-)
HindIII
EcoRI
SYNTHETIC DNA
25
27
29
26
28
30
PCR
HindIII
EcoRI
EcoRI
HindIII
phKM1334LV0

[Figure 13]


Tth111I
EcoRI
phKM1334LV0
Tth111I
EcoRI
phKM1334LV0
PCR
EcoRI
Tth111I
EcoRI
Tth111I
EcoRI
Tth111I
EcoRI
Tth111I
phKM1334LV2

[Figure 14]


Ori
Pmo
hC κ
Sp. β G
A. β G
A.se
Amp
Pmo
NotI
ApaI
hC γ 1
Sp. β G
A. β G
A.se
Pse
dhfr
pKANTEX93
G418
A.tk
A.se
A. β G
Sp. β G
NotI
ApaI
phKM1334HV0
ApaI
NotI
EcoRI
BsiWI
Ori
Pmo
hC κ
Sp. β G
A. β G
A.se
Amp
Pmo
NotI
hKM1334 HV0
ApaI
hC γ 1
Sp. β G
A. β G
A.se
Pse
dhfr
pKANTEX1334HV0
G418
A.tk
A.se
A. β G
Sp. β G
EcoRI
BsiWI
phKM1334LV0
EcoRI
BsiWI
EcoRI
Ori
Pmo
hKM1334 LV0
BsiWI
hC κ
Sp. β G
A. β G
A.se
Amp
Pmo
hKM1334 HV0
hC γ 1
Sp. β G
A. β G
A.se
Pse
dhfr
pKANTEX1334HV0LV0
G418
A.tk
A.se
A. β G
Sp. β G

[Name of Document] Abstract

[Abstract]

[Problems]

An agent for preventing or treating arthritis, a cartilage protecting agent, a joint destruction inhibitor, a synovial membrane growth inhibitor, a diagnostic agent of arthritis and a diagnostic method for judging arthritis are provided.

[Means for Solution]

The present invention provides an agent for preventing or treating arthritis, a cartilage protecting agent, a joint destruction inhibitor and a synovial membrane growth inhibitor comprising an anti-FGF-8 neutralizing antibody as an active ingredient, as well as a diagnostic agent of arthritis comprising an anti-FGF-8 antibody as an active ingredient and a diagnostic method for judging arthritis using the antibody.

[Selected Drawings] None